PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING DOCUMENT TRANSMITTED

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as designated Office

Date of mailing (day/month/year) 14 May 2001 (14.05.01)	
International application No. PCT/EP00/08944	**International filing date** (day/month/year) 11 September 2000 (11.09.00)
Applicant AKZO NOBEL N.V. et al	

The International Bureau transmits herewith the following documents and number thereof:

_________ cop(ies) of priority document(s) (Rule 17.2(a))

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No.: (41-22) 740.14.35	Authorized officer J. Leitao Telephone No.: (41-22) 338.83.38

004020063

Form PCT/IB/310 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
99486 WO		
International application No. PCT/EP 00/08944	International filing date *(day/month/year)* 11/09/2000	(Earliest) Priority Date *(day/month/year)* 10/09/1999
Applicant AKZO NOBEL N.V. et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.
      - [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :
      - [ ] contained in the international application in written form.
      - [ ] filed together with the international application in computer readable form.
      - [ ] furnished subsequently to this Authority in written form.
      - [ ] furnished subsequently to this Authority in computer readble form.
      - [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
      - [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).
3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,
   - [X] the text is approved as submitted by the applicant.
   - [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,
   - [X] the text is approved as submitted by the applicant.
   - [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. ______
   - [ ] as suggested by the applicant.
   - [ ] because the applicant failed to suggest a figure.
   - [ ] because this figure better characterizes the invention.
   - [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
P[illegible]P 00/08944

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/245 A61K39/27 C12N7/00 C12N7/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	OSTERRIEDER N ET AL: "The equine herpesvirus 1 IR6 protein influences virus growth at elevated temperature and is a major determinant of virulence." VIROLOGY, vol. 226, no. 2, 15 December 1996 (1996-12-15), pages 243-251, XP002140685 the whole document ---	1-5
X	MAYR A ET AL.: "Untersuchungen zur Entwicklung eines Lebendimpfstoffes gegen die Rhinopneumonitis (Stutenabort) der Pferde" J. VET. MED. B, vol. 15, 1968, pages 406-418, XP000921173 the whole document ---	1-5
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 January 2001	09/01/2001
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Teyssier, B

1

International Application No
P[illegible]P 00/08944

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	GB 1 570 732 A (PHILIPS ROXANE) 9 July 1980 (1980-07-09) the whole document ---	1-5
X	WO 98 04286 A (RANKIN ANNE M ;SEARLE & CO (US); LUCKOW VERNE A (US); HIPPENMEYER) 5 February 1998 (1998-02-05) page 3, line 17 -page 4, line 1 page 7, line 24 -page 8, line 33 ---	1-5
A	WO 98 06427 A (BAYER AG) 19 February 1998 (1998-02-19) ---	
A	FITZPATRICK D R & STUDDERT M J: "Immunologic ralationships between equine herpesvirus type 1 (equine abortion virus) and type 4 (equine rhinopneumonitis virus)" AMERICAN JOURNAL OF VETERINARY RESEARCH, vol. 45, no. 10, 1 October 1984 (1984-10-01), pages 1947-1952, XP002053536 ---	
A	JACOB R J ET AL.: "Temperature sensitivity of equine herpesvirus isolates: A brief review" SAAS BULLETIN: BIOCHEMISTRY AND BIOTECHNOLOGY, vol. 3, January 1990 (1990-01), pages 124-128, XP000920987 -----	

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 00/08944

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 1570732 A	09-07-1980	US 4110433 A	29-08-1978
		AU 2454077 A	26-10-1978
		BE 853810 A	21-10-1977
		DD 131762 A	19-07-1978
		DE 2717919 A	03-11-1977
		ES 458113 A	16-03-1978
		FR 2348704 A	18-11-1977
		LU 77166 A	12-08-1977
		NL 7704239 A	25-10-1977
		NZ 183907 A	25-10-1979
		PL 197579 A	25-09-1978
		SE 7704514 A	23-10-1977
		SU 700069 A	25-11-1979
WO 9804286 A	05-02-1998	US 5972666 A	26-10-1999
		AU 3977997 A	20-02-1998
		BR 9710604 A	17-08-1999
		CN 1230893 A	06-10-1999
		CZ 9900179 A	14-07-1999
		EP 0956045 A	17-11-1999
WO 9806427 A	19-02-1998	US 5853715 A	29-12-1998
		AU 714418 B	06-01-2000
		AU 4891297 A	06-03-1998
		BR 9711138 A	17-08-1999
		CN 1228029 A	08-09-1999
		EP 0929315 A	21-07-1999

PATENT COOPERATION TREATY

REC'D 04 DEC 2001
WIPO PCT

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 99486 WO	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/EP00/08944	International filing date *(day/month/year)* 11/09/2000	Priority date *(day/month/year)* 10/09/1999
International Patent Classification (IPC) or national classification and IPC A61K39/245		
Applicant AKZO NOBEL N.V. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report
   II ☐ Priority
   III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   IV ☐ Lack of unity of invention
   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   VI ☐ Certain documents cited
   VII ☒ Certain defects in the international application
   VIII ☒ Certain observations on the international application

Date of submission of the demand 10/04/2001	Date of completion of this report 29.11.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Wagner, R Telephone No. +49 89 2399 7357

Form PCT/IPEA/409 (cover sheet) (January 1994)

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
   Description, pages:

   1-14 as originally filed

   Claims, No.:

   1-5 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   - ☐ the description, pages:
   - ☐ the claims, Nos.:
   - ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire international application.

   ☒ claims Nos. 5.

because:

☒ the said international application, or the said claims Nos. 5 (regarding industrial applicability) relate to the following subject matter which does not require an international preliminary examination (*specify*):
see separate sheet

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

   ☐ the written form has not been furnished or does not comply with the standard.

   ☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-5
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-5
Industrial applicability (IA)	Yes:	Claims	1-4

No: Claims

2. Citations and explanations
   see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

<u>Re Item III</u>

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

Claim 5 relates to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

<u>Re Item V</u>

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement.

Reference is made to the following documents:

D1: OSTERRIEDER N ET AL: 'The equine herpesvirus 1 IR6 protein influences virus growth at elevated temperature and is a major determinant of virulence.' VIROLOGY, vol. 226, no. 2, 15 December 1996 (1996-12-15), pages 243-251, XP002140685

D2: MAYR A ET AL.: 'Untersuchungen zur Entwicklung eines Lebendimpfstoffes gegen die Rhinopneumonitis (Stutenabort) der Pferde' J. VET. MED. B, vol. 15, 1968, pages 406-418, XP000921173

For the assessment of the present claim 5 on the question whether it is industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

1. Claim 1 is new (Article 33(2) PCT) because the prior art does not disclose the deposited virus. D1 (abstract) discloses that the modified live vaccine strain RacH is temperature sensitive (i. . the replication at 40°C is 7500-fold reduced

versus that at 37°C). The strain RacH has mutated during the attenuation process (frequent passage on porcine embryonic kidney cells). It is well-known that mutations can be easily induced by mutagens. It is obvious that mutants equivalent to RacH can be obtained by exposing a wild-type strain to a mutagen and by screening for mutations inducing temperature-sensitivity, which is characteristic for the attenuated strains. D1 also cites D2 which discloses the safety and efficacy of RacH as a live attenuated vaccine in horses. Therefore in the absence of substantive evidence (e.g. comparative results) suitable to demonstrate unexpected/advantageous properties associated with the deposited strain ECACC V99061001 disclosed in the present application, the EHV-1 strain of claim 1 and its medical use as a vaccine of dependent claims 2-5 cannot be regarded as inventive over the related mutants and uses disclosed in D1 or D2, contrary to Article 33(3) PCT.

Re Item VII
Certain defects in the international application

1. Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the documents D1 and D2 is not mentioned in the description, nor are these documents identified therein.

Re Item VIII
Certain observations on the international application

1. Claim 1 is not clear (Article 6 PCT) because the formulation "... or progeny thereof.." includes new mutants which are not defined by the deposited strain.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO

AKZO NOBEL NV
VELPERWEG 76
6824 BM ARNHEM
THE NETHERLANDS

NAME AND ADDRESS
OF DEPOSITOR

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: HV-1 TS	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: v99061001
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: [X] A scientific description [ ] A proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depository Authority accepts the microorganism identified under I above, which was received by it on (date of the original deposit)[1]	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depository Authority on (date of the original deposit) and A request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)	
IV. INTERNATIONAL DEPOSITORY AUTHORITY	
Name: Dr P J Packer Address: ECACC CAMR Port n Down Salisbury SP4 OJG	Signature(s) of person(s) having the power to represent the International Depository Authority or of authorized officials(s): PJPacker Date: 9/9/00

1 Where Rule 6.4(d) applies, such dat is th dat on which the status of int rnational d positary authority was acquired

Form BP/4 (sole page) 1991

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/08944

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/245 A61K39/27 C12N7/00 C12N7/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	MAYR A ET AL.: "Untersuchungen zur Entwicklung eines Lebendimpfstoffes gegen die Rhinopneumonitis (Stutenabort) der Pferde" J. VET. MED. B, vol. 15, 1968, pages 406-418, XP000921173 the whole document ---	1-5

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 January 2001	09/01/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Teyssier, B

1

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GB 1 570 732 A (PHILIPS ROXANE) 9 July 1980 (1980-07-09) the whole document	1-5
X	WO 98 04286 A (RANKIN ANNE M ;SEARLE & CO (US); LUCKOW VERNE A (US); HIPPENMEYER) 5 February 1998 (1998-02-05) page 3, line 17 -page 4, line 1 page 7, line 24 -page 8, line 33	1-5
A	WO 98 06427 A (BAYER AG) 19 February 1998 (1998-02-19)	
A	FITZPATRICK D R & STUDDERT M J: "Immunologic ralationships between equine herpesvirus type 1 (equine abortion virus) and type 4 (equine rhinopneumonitis virus)" AMERICAN JOURNAL OF VETERINARY RESEARCH, vol. 45, no. 10, 1 October 1984 (1984-10-01), pages 1947-1952, XP002053536	
A	JACOB R J ET AL.: "Temperature sensitivity of equine herpesvirus isolates: A brief review" SAAS BULLETIN: BIOCHEMISTRY AND BIOTECHNOLOGY, vol. 3, January 1990 (1990-01), pages 124-128, XP000920987	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 00/08944

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 1570732 A	09-07-1980	US 4110433 A	29-08-1978
		AU 2454077 A	26-10-1978
		BE 853810 A	21-10-1977
		DD 131762 A	19-07-1978
		DE 2717919 A	03-11-1977
		ES 458113 A	16-03-1978
		FR 2348704 A	18-11-1977
		LU 77166 A	12-08-1977
		NL 7704239 A	25-10-1977
		NZ 183907 A	25-10-1979
		PL 197579 A	25-09-1978
		SE 7704514 A	23-10-1977
		SU 700069 A	25-11-1979
WO 9804286 A	05-02-1998	US 5972666 A	26-10-1999
		AU 3977997 A	20-02-1998
		BR 9710604 A	17-08-1999
		CN 1230893 A	06-10-1999
		CZ 9900179 A	14-07-1999
		EP 0956045 A	17-11-1999
WO 9806427 A	19-02-1998	US 5853715 A	29-12-1998
		AU 714418 B	06-01-2000
		AU 4891297 A	06-03-1998
		BR 9711138 A	17-08-1999
		CN 1228029 A	08-09-1999
		EP 0929315 A	21-07-1999

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 March 2001 (15.03.2001)

PCT

(10) International Publication Number
WO 01/17553 A1

(51) International Patent Classification[7]: A61K 39/245, 39/27, C12N 7/00, 7/06

(21) International Application Number: PCT/EP00/08944

(22) International Filing Date: 11 September 2000 (11.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
99202933.0 10 September 1999 (10.09.1999) EP

(71) Applicant *(for all designated States except US)*: **AKZO NOBEL N.V.** [NL/NL]; Velperweg 76, NL-6824 BM Arnhem (NL).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: **PATEL, Jay, R.** [GB/GB]; Highfield Cottage, 51 Westdrive, Caldecote, Cambridge CB3 7NY (GB).

(74) Agent: **OGILVIE-EMANUELSON, C., M.**; P.O. Box 20, NL-5340 BH Oss (NL).

(81) Designated States *(national)*: AE, AG, AL, AU, BA, BB, BG, BR, BZ, CA, CN, CR, CU, CZ, DM, DZ, EE, GD, GE, HR, HU, ID, IL, IN, IS, JP, KP, KR, LC, LK, LR, LT, LV, MA, MG, MK, MN, MX, MZ, NO, NZ, PL, RO, RU, SG, SI, SK, SL, TR, TT, UA, US, UZ, VN, YU, ZA.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*
— *With (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/17553 A1

(54) Title: EQUINE HERPES VIRUS TEMPERATURE SENSITIVE MUTANT AND LIVE VACCINE THEREOF

(57) Abstract: The present invention relates to an equine abortion virus (EHV-1) mutant which is temperature sensitive at the body temperature of the host animal, more specifically at a temperature of 38.5 °C or higher. The temperature sensitive mutant can be used in vaccination to protect susceptible animals against EHV-1 infection. The invention furthermore relates to live vaccines derived from said mutant

EQUINE HERPES VIRUS TEMPERATURE SENSITIVE MUTANT AND LIVE VACCINE THEREOF

The present invention relates to an equine abortion virus mutant, a process for the preparation of said mutant, use of said mutant and live vaccines derived from said mutant.

Equine abortion virus (EHV-1), a herpes virus, is a major equine pathogen responsible for viral-induced abortion, neurological disease such as paresis, infections of the upper respiratory tract, and neonatal foal disease (NFD). NFD results from close to term transplacental infection of fetuses, which are born weak with severe respiratory disease and some with jaundice due to liver infection by EHV-1. These animals usually die within a few days after birth. Equine rhinopneumonitis virus (EHV-4) is the major cause of acute respiratory tract disease ("rhinopneumonitis") and infects most horses during their first two years of life. Rhinopneumonitis is characterized by fever, anorexia, and profuse serous nasal discharge that later becomes mucopurulent. On rare occasions EHV4 infection causes abortion in pregnant mares. Furthermore EHV1 and EHV4 establish persistent, lifelong latent infections. Upon reactivation the viruses cause recurrent disease, accompanied by virus shedding and transmission to other animals.

Control of equine herpes virus infection and their diseases remain inadequate, in particular against EHV1 mediated abortions, paresis and neonatal foal disease resulting from close to term transplacental infection of foetus. Although inactivated as well as modified live vaccines are available, neither vaccine appears to block infection sufficiently, nor do they prevent the establishment of latency by wild-type virus. Hence there is a great need for safe vaccines with improved protection against field infections of these viruses, particularly against infections caused by EHV1.

The present invention provides for such vaccines.

In a first aspect the present invention provides for an EHV-1 Ts mutant as deposited at the European Collection of Animal Cell Culture (ECACC), Salisbury, Wiltshire SP4 0JG, UK on 10 June 1999 under accession number V99061001, and progeny thereof.

The EHV-1 Ts mutants according to the invention are furthermore phenotypically characterized in that

•they form small plaques when grown on several horse cell lines,

• they have lost their ability to grow on rabbit kidney cells, in particular RK13 cells, and

•they are limited in their ability to cause viraemia (that is, they are able to ;

The EHV-1 Ts mutants according to the invention have the advantage that replication is restricted to the upper respiratory tract of conventional equidae with no or limited ensuing viraemia. The Ts mutants are safe for pregnant mares while giving rise to significant immune stimulation following growth in the upper respiratory tract. The Ts mutants are not readily back-passaged form animal to animal thus limited in their potential for transmission and reversion.

For the purpose of this invention "progeny" is defined to include also all strains obtained by further serial passage of the deposited EHV-1 Ts mutant.

For the purpose of this invention, a temperature sensitive mutant is defined as a mutant virus which has an impaired growth at or above a certain temperature at which the wild type has a normal growth. The EHV-1 Ts mutants according to the present invention are characterized in that they are temperature sensitive at the body temperature of the host animal. The EHV-1 Ts mutants of the present invention do not replicate above a temperature of 38.5 to 39.0°C. Preferably the EHV-1 Ts mutants according to the invention do not replicate at a temperature of 38.5°C.

For the purpose of this invention, small plaques are defined as plaques that are at least half to one third the size of the plaques formed by the wild-type parent strain in equine cells.

For the purpose of this invention the "limited abilty to cause viraemia" is defined as the ability to cause no or low grade (that is, just detectable) vireamia for 1 to 3 or 4 days in some animals with respect to the ability of the parent strain to cause viraemia.

Temperature sensitive EHV-1 mutants according to the invention can be obtained by treatment of infected bovine, equine or other permissive cell cultures at 34°C with non-toxic concentrations of a mutagens such as 5-bromo-2-deoxy uridine, azacytidine and the like during viral replication in vitro, followed by biological cloning of progeny virus from said treated cultures in bovine or equine or other permissive cell lines.

The favorable properties of the Ts-mutants according to the invention makes them very suitable for use in the preparation of a vaccine. Thus, in a second aspect the present invention provides for a composition, in particular a vaccine composition, comprising an EHV-1 Ts-mutant according to the invention, and a pharmaceutically acceptable carrier or vehicle. More specifically, a (vaccine) composition according to the invention comprises the EHV1 Ts-mutant deposited at the ECACC, Salisbury, UK having accession number V99061001 and/or progeny thereof. Pharmaceutical acceptable carriers or vehicles that are suitable for use in a vaccine according to the invention are sterile water, saline, aqueous buffers such as PBS and the like. In addition a vaccine according to the invention may comprise other additives such as adjuvants, stabilizers, anti-oxidants and others.

The vaccine compositions according to the invention are safe and can be used to protect the equidae clinically and virologically against infections with EHV-1 and to protect against virus-induced abortions and paresis. In addition the vaccine according to the invention was found to stop trans-placental infection, thus protecting the newborn foal from the effects of neonatal foal disease. The vaccine composition according to the present invention can be administered not only to horses but also to other animals that are susceptible to EHV-1 infection such as donkeys, zebra's and the like. Cattle which have been reported to be susceptible to EHV-1 and EHV-4 infection can also be treated with the vaccine according to the invention.

It was furthermore surprisingly found that vaccines comprising an EHV-1 Ts-mutant according to the invention not only protect against EHV-1 infections but also against the disease and the associated virus shedding following EHV-4 infection. Thus such a vaccine can be useful to obtain cross-protection in the vaccinated equidae. Said vaccines give rise to improved protection thus effectively blocking infection with wild-type viruses.

Vaccine compositions according to the invention can be prepared following standard procedures. A vaccine according to the invention preferably is a live vaccine. For the preparation of the live vaccine, the seed virus of the EHV-Ts mutant can be grown on a cell culture, such as primary or secondary bovine kidney or equine cells. The viruses thus grown can be harvested by collecting the tissue cell culture fluids and/or cells. Optionally, during harvesting the yield of the viruses can be promoted by techniques that improve the liberation of the infective particles from the growth substrate, e.g. sonication. The live vaccine may be prepared in the form

of a suspension or may be lyophilized.

Pharmaceutical acceptable carriers that are suitable for use in a vaccine according to the invention are sterile water, saline, aqueous buffers such as PBS and the like. In addition a vaccine according to the invention may comprise other additives such as adjuvants, stabilizers, anti-oxidants and others.

Suitable stabilizers are for example carbohydrates including sorbitol, mannitol, starch, sucrose, dextran and glucose, proteins and degradation products thereof including but not limited to albumin and casein, protein-containing agents such as bovine serum or skimmed milk, and buffers including but not limited to alkali metal phosphates. In lyophilized vaccine compositions it is preferable to add one or more stabilizers.

Suitable adjuvants include but are not limited to aluminum hydroxide, phosphate or oxide, amphigen, tocopherols, monophosphenyl lipid A, muramyl dipeptide, oil emulsions, glucans, carbomers, block-copolymers, cytokines and saponins such as Quil A. The amount of adjuvant added depends on the nature of the adjuvant itself.

EHV-1 Ts mutants according to the invention are preferably administered to conventional, seronegative animals varying in ages from a few days to several years, including those in-foal. The vaccine can be administered to the animals via non-parenterally administration routes, including but not limited to intradermal, oral, spraying, aerosol, intra-ocular, and intranasal administration. Alternatively, the vaccine can be administered via parenteral administration routes. Preferably the vaccine is administered intradermally or intranasally.

In general the EHV-1 Ts mutant virus is administered in an amount that is effective to induce protection against EHV-1 infection. The dose generally will depend on the route of administration, the time of administration, as well as age, health and diet of the animal to be vaccinated. The virus can be administered in an amount between 10^2 and 10^9 pfu/dose per animal, preferably between 10^3 and 10^5 pfu/dose and more preferably at 10^4 pfu/dose per animal.

The vaccines according to the invention also may be given simultaneously or concomitantly with other live or inactivated vaccines. These additional vaccines can be administered non-parenterally or parenterally. Preferably the additional vaccines are recommended for parenteral administration.

EXAMPLES

1. *Isolation and characterization of a temperature sensitive EHV-1 mutant strain TS C147*

Just confluent, day-old 75 cm^2 monolayers of equine dermal (ED) cells were infected at m.o.i. of 0.001 with EHV-1. Inoculum (2.0 ml) was adsorbed (1 hour, 37°C), removed and monolayers were washed with PBS and then re-fed with tissue culture medium (25 ml) containing 40 µg/ml of 5-bromo-2-deoxy uridine and incubated at 34°C. At maximum CPE (7 days post inoculation), the culture was harvested (frozen at -40°C and then thawed at 37°C), dialyzed overnight at 4°C against PBS, titrated for EHV-1 infectivity in ED cells at 37°C and subsequently cloned at 34°C in ED cells grown in 96-well microtitration plates. Wells with single EHV-1 focus were identified, allowed to grow to maximum CPE and then a small (20 µl out of 200 µl total) sample used for phenotyping at permissive (34°C) and restrictive (39°C) temperatures using ED cells. Temperature sensitive clones were further passaged in Bovine Kidney cells, strain JCK (Jay's Calf Kidney- Intervet's strain) to produce the master and working seeds.

2. *Temperature sensitive phenotype of EHV-1 strain TS C147*

EHV-1 TS C147 strain at Master Seed Virus (MSV)+1°level was titrated in parallel in Bovine Embryo Lung (strain BEL_{26} – Intervet's strain), Bovine Kidney (strain Jay's Calf Kidney, JCK – Intervet's strain), Equine Dermal (ED) cells, Equine Dermal Clone W48 C10 (ED W48 C10 – Intervet's strain), and Equine Dermal Clone W7 C5 (ED W7 C5 – Intervet's strain) at 37°C and 38.5°C. Virus at MSV+1° passage level failed to grow at 38.5°C. Results are given in Table 1.

3. *EHV-1 strain TS C147 has EHV-4 like characteristics*

A parameter for the differentiation between EHV-1 and EHV-4 is their ability to replicate in rabbit kidney (RK13) cells. EHV-1 strains replicate well in RK13 cells but the cells are refractory to EHV-4 strains. EHV-1 strain TS C147 at MSV+1° level, its wild type parent EHV-1 strain, EHV-1 strain deficient in immediate early gene (EHV-1 IE), pathogenic EHV-1 strain CHLi and a field EHV-4 isolate were titrated in parallel at 37°C in RK13 cells and Equine dermal (ED) cells. Results given in Table 2 show that the 4 EHV-1 strains, including strain TS C147 and

EHV-4 strain replicated in ED cells but EHV-1 strain TS C147 and EHV-4 strain did not grow in RK13 cells.

TABLE 1: Relative titers of EHV-1 strain TS C147 at 37°and 38.5° in various bovine and equine cell strains

Titers (log_{10} $TCID_{50}$/ml) at 37°C and 38.5°C in various bovine and equine cell types		Virus Passage level TSC147 MSV+1°
BEL_{26}:	37°C	5.2
	38.5°C	<1.1
JCK:	37°C	5.4
	38.5°C	<1.1
ED:	37°C	5.4
	38.5°C	<1.1
ED W48 C10:	37°C	5.7
	38.5°C	<1.1
ED W7 C5:	37°C	5.7
	38.5°C	<1.1

a) Titers after 5 days' incubation; Titters given as <1.1 log_{10} $TCID_{50}$/ml represent no EHV-1 foci detected in 4 x 200µl of the lowest (10^{-1}) dilution of the virus tested in the titration

Table 2: Ability of rabbit kidney cells to support replication of EHV-1 strain TS C147

Virus	Relative titer (log_{10} $TCID_{50}$/ml) at 37°C in RK13 & ED cells	
	RK13 cells	ED cells
EHV-1 TS C147 MSV+1°	<1.1[a]	5.7
EHV-1 040	5.7	5.7
EHV-1 IE	6.0	6.2
EHV-1 CHLi	5.7	6.0
EHV-4	<1.1	3.7

a = Titers given as <1.1 log_{10} $TCID_{50}$/ml represent no EHV-1 CPE detected in 4 x 200µl of the lowest dilution (10^{-1}) in the titration.

4. *Clinical and virological protection of conventional ponies against infections by EHV-1 and EHV-4*

Of 29 conventional ponies with low or no EHV-1 neutralizing (VN) antibody, 15 were vaccinated intranasally (IN) with a dose of 5.3 log_{10} $TCID_{50}$ of strain EHV-1 TS C147 while 14 ponies were left unvaccinated to serve as unvaccinated control. About a month following a single IN vaccination, 8 vaccinated and 8 unvaccinated (control) ponies were challenged IN with a field strain of EHV-1 while a group of 7 EHV-1 vaccinated and 6 control animals were challenged IN with a recent field isolate of EHV-4. Following vaccination and challenge, animals were monitored for clinical reactions, virus shedding in nasal mucus, infected leukocytes (viraemia) and EHV-1 neutralizing antibody.

Vaccine virus grew to low titers (nasal mucus peak titers 1.5 to 3.0 log_{10} $TCID_{50}$/ml) for 1 to 8 days in 11/15 ponies and also resulted in low-grade (just detectable) leukocyte viraemia for 1 to 4 days in 7 of 15 animals. However all 15 ponies seroconverted. In contrast no EHV-1 was recovered from the nasal mucus or the blood of 14 control ponies monitored daily for 10 or 14 days respectively and the animals remained seronegative to EHV-1 until after challenge infection. A similar level of pyrexia was seen in 10 animals in each of the two (vaccinated and control) groups. These findings are summarized in Table 3.

Following intranasal EHV-1 challenge, there was a significant reduction in virus excreted in nasal mucus by the vaccinated ponies relative to that recovered from the control animals. Similarly a single vaccination prevented leukocyte viraemia in 7 of 8 ponies while one pony was just virus positive for 1 day. In contrast, however all 8 unvaccinated ponies became viraemic, 7 for 3 to 4 days and 1 for 1 day. All 8 control ponies became moderately to highly febrile for 1 to 6 days but all 8 vaccinated animals remained normal. None of the 8 vaccinated animals responded anamnestically to the challenge infection while all 8 control animals responded with a significant EHV-1 neutralizing antibody. These findings are summarized in Table 4.

Following intranasal EHV-4 challenge, virus was recovered from the nasal mucus of one of 7 vaccinated ponies on one occasion but all 6 control ponies excreted virus at a significantly higher titer for 2 to 3 days, with one exception. None of the 7 EHV-1 vaccinated ponies became viraemic in contrast to 3 of 6 control ponies for 1-3 days. EHV-4 challenge infection resulted in pyrexia in 3 of 6 control animals for 2 to 3 days but none of the 7 vaccinated ponies were affected. There was a slight (15 to 20 expirations/minute) increase in respiration rate in 4 of 7 vaccinated and 5 of 6 control animals for 1 to 3 and 2 to 6 days respectively. These findings are summarized in Table 5.

TABLE 3: **Results after vaccination**

Result – No +ve/No Total (Peak activity range & duration)

Parameter	Vaccinated	Control
Virus shedding in mucus	11/15 (1.5-3.0 $\log_{10}$ $TCID_{50}$/ml, 1-8 days)	0/14
Leukocyte viraemia	7/15 (low grade, 1-4 days)	0/14
Seroconversion	15/15	0/14
Pyrexia (=38.5°C) (Between days 1-10)	10/15 (38.5-39.3, 1-3 days)	10/14 (38.5-38.8, 1-3 days)

TABLE 4: **After EHV-1 challenge**

Result – No +ve/No Total (Peak activity range & duration)

Parameter	Vaccinated	Control
Virus shedding in mucus	5/8 (1.5 $\log_{10}$/ml, 1-2 days)	8/8 (2.2-3.4 $\log_{10}$ $TCID_{50}$/ml, 4-6 days)
Leukocyte viraemia	1/8 (low grade, 1 day)	8/8 (3-4 days; 1 day for 1 animal)
EHV-1 (VN) antibody rise)	0/8 (=4-fold rise)	8/8
Pyrexia	0/8	8/8 (38.9-41.0, 1-6 days)
Respiration	3/8 (15-20 expirations/min, 1 day)	2/8 (15-20 expirations/min 1 day)

TABLE 5: Results after EHV-4 challenge

Result – No +ve/No Total (Peak activity range & duration)

Parameter	Vaccinated	Control
Virus shedding in mucus	1/7 (1.5 log_{10}/ml, 1 day)	6/6 (1.5-3.7 log_{10} $TCID_{50}$/ml, 1-3 days)
Leukocyte viraemia	0/7	3/6 (1-3 days)
EHV-1 (VN) antibody rise)	1/7 (=4-fold rise)	5/6
Pyrexia	0/7	3/6 (38.6-38.8, 2-3 days)
Respiration	4/7 (15-20 expirations/min, 1-3 days)	5/6 (15-20 expirations/min 2-6 days)

5. *Protection of equidae against paresis and abortions due to EHV-1 infection*

Of 12 pregnant mares with low or no EHV-1 neutralizing (VN) antibody, 6 were vaccinated intranasally (IN) at about 6 months of gestation and then all 12 mares challenged IN with a pathogenic strain of EHV-1 at the critical stage of gestation for EHV-1 abortions namely about 9 months of gestation. Following vaccination and challenge, animals were monitored for clinical reactions, virus shedding in nasal mucus, infected leukocytes (viraemia) and EHV-1 neutralizing antibody.

Although no vaccine virus was recovered from nasal mucus from any of 6 vaccinated mares, low grade, transient (1 to 3 days) viraemia was detected in 5 of 6 mares and all 6 animals seroconverted with significant VN antibody to EHV-1. None of the 6 control mares, monitored in parallel to vaccinated animals for 10 to 14 days, yielded EHV-1 from nasal mucus or leukocytes but 1 of 6 animals seroconverted some 2½ months later. These findings are summarised in Table 6.

Following challenge, there was a significant (2 out of 6 compared to 5 of 6 and 1.5 to 1.7 log_{10} $TCID_{50}$/ml for 1-2 days compared to 2.4 to 3.7 log_{10} $TCID_{50}$/ml for 1-6 days) reduction in virus excreted in nasal mucus by the vaccinated mares. Similarly none of 6 vaccinated mares became viraemic in contrast to 5 of 6 unvaccinated control mares. In the control group 5 of 6 mares became

febrile for 1 to 5 days, 3 also developed paresis accompanied by severe jaundice and disintegration of the cervical plug in 2 mares with signs of foetal ejection. One of the two animals died while the 2[nd] had to be euthanased *in extremis*. Both animals carried dead foals. Three further mares aborted. Foetal tissues from all 5 foetuses were EHV-1 positive. In contrast however all 6 vaccinated mares foaled normally. The only clinical reaction observed in vaccinated mares was transient (1 day) pyrexia in one of 6 mares. The control mare which foaled normally had in fact seroconverted just prior to challenge. These findings are summarised in Table 7.

TABLE 6: **Results after vaccination**

Result – No +ve/No Total (Peak activity range & duration)

Parameter	Vaccinated (group 1)	Control (group 2)[a]
Virus shedding in mucus	0/6	Not monitored[a]
Leukocyte viraemia	5/6 (low, 1-3 days)	0/6
Pyrexia	1/6 (1 day)	Not monitored[a]
Seroconversion	6/6 (Month onwards after vaccination)	1*/6 (About 3 months after vaccination)

a = Not monitored because animals kept in isolation away from the vaccinated group

TABLE 7: Results after challenge

Result – No +ve/No Total (Peak activity range & duration)

Parameter	Vaccinated (group 1)	Control (group 2)
Virus shedding in mucus	2/6 (1.5-1.7 $\log_{10}$ $TCID_{50}$/ml, 1-2 days)	5/6 (2.4-3.7 $\log_{10}$ $TCID_{50}$/ ml, 1-6 days)
Leukocyte viraemia	0/6	5/6
EHV-1 VN antibody rise	0/6	5/6†
Pyrexia	1/6 (1 day)	5/6 (1-5 days)
Paresis	0/6	3/6 (Terminal in 2 mares)
Jaundice	0/6	2/6
Death	0/6	2/6 (1 died, 1 euthanased *in extremis* with severe paralysis jaundice and rapid decrease in body temperature
Abortion	0/6	5/6

† Control mare was seronegative in 3-monthly bleeds after vaccination of group 1 mares but seroconverted prior to challenge.

6. *Safety of EHV-1 TS C147 in pregnant mares*

Four mares at about 9 months of gestation (critical stage for EHV-1 abortions) were inoculated by the natural route with 10 times the protective dose and monitored for abortions. Results given in Table 8 show that all 4 mares seroconverted to EHV-1, one of 4 mares became transiently viraemic but foaled normally. Three of 4 foals were EHV-1 VN antibody negative in blood samples collected before suckling the respective dam while one foal was VN antibody positive due to colostrum intake (born between monitoring intervals in the early hours). These results are summarised in Table 8.

TABLE 8: An overdose safety for pregnant mares at the critical stage of gestation for EHV-1 abortions

Mare No	Shedding in nasal mucus	Viraemia	VN antibody to EHV-1 At dosing & 3 wks later		Foaling & antibody[a]	
13	c	-ve	<2.0	3.5	Normal	<2.0
14A	c	+ve (3 days)	<2.0	6.0	Normal	4.0[b]
15	c	-ve	<2.0	5.0	Normal	<2.0
16	c	-ve	<2.0	6.0	Normal	<2.0

a = EHV-1 neutralizing antibody at birth
b = Born between monitoring intervals in early hours and the foal was bled at least 3 hours after birth
c = Pending, ie to be done

7. *No transmission of EHV-1 TS C147 between target species.*

A back-passage study was performed in EHV-naïve (all types) weaned foals (specific pathogen free, SPF foals).

Two SPF foals were inoculated intranasally (IN) with 10 times protective dose of EHV-1 strain TS C147 at Master Seed Virus+1° passage level and virus positive nasal mucus collected over several days used to similarly infect a further pair of SPF foals. After IN inoculation, foals were monitored for (i) virus shedding in nasal mucus, (ii) clinical reactions and (iii) seroconversion to EHV-1.

Foals given EHV-1 strain TS C147 at MSV+1° level excreted virus in nasal mucus and seroconverted. However, a pool of virus positive nasal mucus samples failed to infect a further pair of EHV-naïve foals as judged from the failure to recover EHV-1 from their nasal mucus and the absence of seroconversion to EHV-1. The results were confirmed by repeating the study with a further 4 SPF foals, 2 inoculated with MSV+1° followed by a further 2 given virus positive nasal mucus from the first two foals. Results are summarised in Tables 9 and 10.

TABLE 9: Backpassage of EHV-1 strain TS C147 in EHV-naïve foals

(i) <u>PASSAGE ONE</u>: Foals 1 & 2 inoculated intranasally with EHV-1 TS C147 (10x protective dose) at MSV+1° level.

Parameter	Result (+/-, range & duration)
Virus shedding in nasal mucus	2/2 (1.7-5.0 $\log_{10}$ $TCID_{50}$/ml, 5-6 days)
Seroconversion to EHV-1	2/2 (bleed 2 weeks after inoculation & by CF test)

(ii) <u>PASSAGE TWO</u>: Foals 5 & 6 inoculated intranasally with virus positive nasal mucus from foals 1 & 2

Parameter	Result (+/-, range & duration)
Virus shedding in nasal mucus	0/2
Seroconversion	0/2

TABLE 10: Backpassage of EHV-1 strain TS C147 in EHV-naïve foals

(i) PASSAGE ONE: Foals 7 & 8 inoculated intranasally with EHV-1 TS C147 (10x protective dose) at MSV+1° level.

Parameter	Result (+/-, range & duration)
Virus shedding in nasal mucus	2/2 (1.5-3.7 $\log_{10}$ $TCID_{50}$/ml, 4-8 days)
Seroconversion to EHV-1	2/2 (bleed 2 weeks after inoculation & by CF test)

(ii) PASSAGE TWO: Foals 9 & 10 inoculated intranasally with virus positive nasal mucus from foals 7 & 8

Parameter	Result (+/-, range & duration)
Virus shedding in nasal mucus	0/2
Seroconversion to EHV-1	0/2

Claims

1. A temperature-sensitive (Ts) mutant of Equine abortion virus (EHV-1) characterized in that the virus is the EHV1 Ts-mutant deposited at the ECACC under Accession No. V99061001, or progeny thereof.

2. Pharmaceutical composition comprising a temperature-sensitive mutant according to claim 1 and a pharmaceutical acceptable vehicle or carrier.

3. Vaccine for the prevention and/or treatment of EHV-1 infections in equidae comprising a temperature-sensitive mutant virus according to claim 1 and a pharmaceutically acceptable carrier or diluent.

4. Use of a temperature sensitive mutant according to claim 1 in the manufacture of a vaccine for the prevention and/or treatment of EHV-1 infections.

5. A method for the immunization of an animal against EHV-1 infection comprising administering to said animal a vaccine according to claim 4.

PCT/EP00/08944

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO

AKZO NOBEL NV
VELPERWEG 76
6824 BM ARNHEM
THE NETHERLANDS

NAME AND ADDRESS
OF DEPOSITOR

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
HV-1 TS	V99061001

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

[X] A scientific description

[ ] A proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depository Authority accepts the microorganism identified under I above, which was received by it on (date of the original deposit)[1]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depository Authority on (date of the original deposit) and A request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)

IV. INTERNATIONAL DEPOSITORY AUTHORITY

Name: Dr P J Packer	Signature(s) of person(s) having the power to represent the International Depository Authority or of authorized officials(s):
Address: ECACC CAMR Porton Down Salisbury SP4 OJG	Date: PJPacker 9/9/00

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1991

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/08944

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/245 A61K39/27 C12N7/00 C12N7/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	OSTERRIEDER N ET AL: "The equine herpesvirus 1 IR6 protein influences virus growth at elevated temperature and is a major determinant of virulence." VIROLOGY, vol. 226, no. 2, 15 December 1996 (1996-12-15), pages 243-251, XP002140685 the whole document ---	1-5
X	MAYR A ET AL.: "Untersuchungen zur Entwicklung eines Lebendimpfstoffes gegen die Rhinopneumonitis (Stutenabort) der Pferde" J. VET. MED. B, vol. 15, 1968, pages 406-418, XP000921173 the whole document ---	1-5

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
3 January 2001	09/01/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Teyssier, B

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/08944

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GB 1 570 732 A (PHILIPS ROXANE) 9 July 1980 (1980-07-09) the whole document ---	1-5
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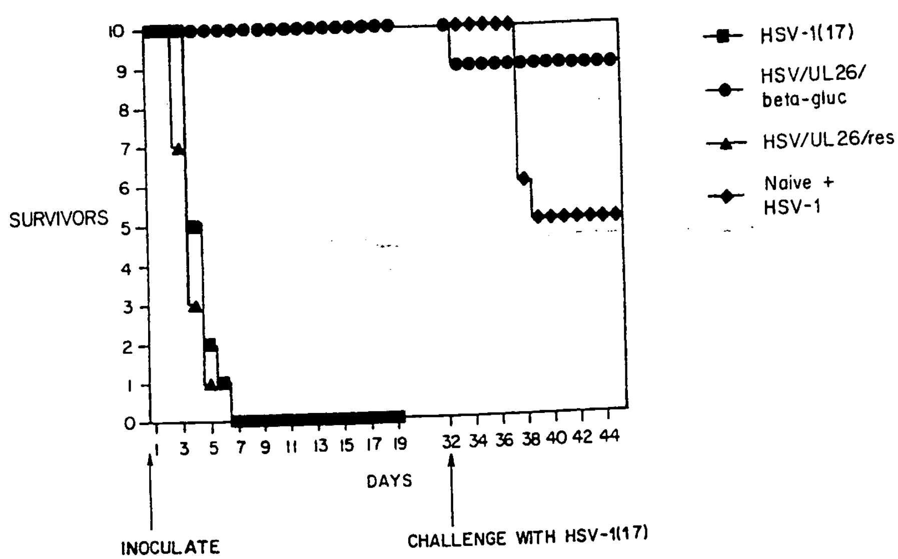

(57) Abstract

A vaccine is described which comprises an assembly-deficient herpesvirus. The mutant herpesvirus is capable of infecting and undergoing DNA replication in the cells of a susceptible mammal, but is defective in capsid assembly and formation of mature virion particles. The assembly-deficient herpesvirus is avirulent and capable of generating a protective immune response in a vaccinated mammal.

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ASSEMBLY-DEFICIENT HERPESVIRUS VACCINE

FIELD OF THE INVENTION

This invention is in the field of viral vaccines, and specifically relates to the generation of assembly-deficient mutant herpesviruses, vaccines comprising assembly-deficient mutant herpesviruses, and methods for the production and manufacture of assembly-deficient herpesvirus vaccines.

BACKGROUND OF THE INVENTION

There is a great need for therapies for the treatment of viral diseases. While antiviral drugs such as zidovudine, used in the treatment of human immunodeficiency virus (HIV), and drugs such as ganciclovir, acyclovir, and foscarnet are used in the treatment of herpesvirus infections, significant side effects often limit their effectiveness. The selection and spread of drug-resistant viruses also limits the effectiveness of small molecular weight antiviral drugs. This is a particularly significant problem for drugs targeted against RNA viruses such as HIV, which have a relatively high mutation rate compared to most DNA viruses.

Antiviral vaccines are a viable alternative to postinfection antiviral drug treatments. Ideally, antiviral vaccines protect against primary disease and recurring infections. Efficacy against a particular disease is crucial to the development of a vaccine strategy. Regulatory concerns, particularly related to the safety of vaccines intended for prophylactic use in healthy individuals, must also be considered.

While herpesvirus vaccines have been an active area of both academic and commercial interest, induction of a good,

protective immune response in humans has been challenging [R. L. Burke, *Current Status of HSV Vaccine Development*, in **The Human Herpesviruses**, 367-379, (B. Roizman, R. J. Whitley and C. Lopez, eds. 1993)]. Live virus vaccines have the risk of establishing latency and reactivating. Live virus vaccines also have the potential of recombining with natural isolates.

Attenuated recombinant viruses and subunit vaccines have been investigated to avoid these risks. Meignier et al describe a recombinant virus resulting from the removal of a region of herpes simplex virus type 1 (HSV-1) required for virulence and the insertion of herpes simplex virus type 2 (HSV-2) glycoprotein genes [J. Infect. Dis., 158:602-614 (1988)]. The viruses had reduced pathogenicity and induced immunity in a number of animal models.

More recently, recombinant herpes simplex viruses with deletions in essential immediate early or early genes have been described. These recombinant viruses are described as being efficacious in inducing immunity and reducing acute replication and establishment of latency of the challenged wild-type virus in mice. Nguyen et al describe replication-defective mutants of HSV-1 that have mutations in the essential genes encoding infected cell protein 8 ("ICP8") or ICP27 [J. Virol. 66:7067-7072 (1992)]. The ICP8 mutant (d301) expresses the products of the α and β genes while the ICP27 mutant (n504) expresses the products of the α, β, and γ_1 genes in the cells that the viruses can infect. Both viruses induced antibody responses that were lower than parental (KOS 1.1) virus, but the level induced by the ICP27 mutant was higher than that induced by infection with the ICP8 mutant. Morrison and Knipe later demonstrated that injection of these viruses protected mice against development of encephalitis and keratitis, and decreased the primary replication of virulent challenge

virus [J. Virol. 68:689-696 (1994)]. WO95/18852 describes similar replication-defective herpesvirus mutants and WO94/03207 describes vaccines based on these mutants.

Another recombinant virus has been described that has a deletion in the glycoprotein H (gH) coding region [Forrester et al, J. Virol. 66:341-348 (1992); WO92/05263]. This virus forms virions after infection of non-helper cells, but the viruses fail to infect in a subsequent round. Inoculation of mice with the gH deletion virus resulted in a more rapid clearance of the wild-type challenge virus compared to vaccination with chemically-inactivated virus [Farrell et al, J. Virol. 68:927-932 (1994)]. Inoculation of guinea pigs with the gH deleted recombinant virus resulted in reduced primary vaginal disease and reduced recurrences [McLean et al, J. Infect. Dis. 170:1100-1109 (1994)].

Most viruses encode proteinases that function in the processing of viral proteins during infection [W. G. Dougherty and B. L. Semler, Microbiological Reviews, 57:781-822 (1993)]. Biological and biochemical studies have shown that HSV-1 possesses a proteinase that can process another viral protein, the capsid assembly protein (also known as p40, ICP35 and VP22a). Similar proteinases are encoded in the genome of other members of the Herpesviridae. This family of DNA viruses includes HSV-1, HSV-2, human and simian cytomegalovirus (HCMV, SCMV), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), human herpesvirus types -6, -7, and -8 (HHV-6, HHV-7, and HHV-8), pseudorabies virus (PRV), bovine herpesvirus (BHV), equine herpesvirus (EHV), and rhinotracheitis virus, among others.

Early work by Preston et al, [J. Virol. 45:1056-1064 (1983)] showed that a temperature-sensitive (ts) mutant in HSV-1 (ts1201) failed to cleave the capsid assembly protein to its lower molecular weight forms at the nonpermissive

temperature. This mutant also failed to package viral DNA. By marker rescue, the defect was mapped to a region of the genome in what is now known as the UL26 open reading frame (ORF) [McGeoch et al, J. Gen. Virol. 69:1531-1574 (1988)]. Subsequent analysis showed that two transcripts initiate in the UL26 region, a primary transcript of about 2.1 kb which encodes a protein of 635 amino acids, and a more abundant transcript which is initiated within the UL26 ORF, about 1000 nucleotides 3′ of the primary transcript initiation. This smaller transcript encodes a predicted protein of 329 amino acids and is 3′ coterminal with the larger 80 kDa ORF encoded by the larger transcript [F. Y. Liu and B. Roizman. J. Virol. 65:206-212 (1991)]. The defect in the ts1201 mutant maps in the 5′ region of the longer transcript which has been shown to encode a proteinase activity in HSV-1 [F. Y. Liu and B. Roizman. J. Virol. 65:5149-5156 (1991)] or in simian cytomegalovirus [Welch et al, Proc. Natl. Acad. Sci. USA. 88:10792-10796 (1991)].

Superinfection/transient expression [F. Y. Liu and B. Roizman. J. Virol. 65:5149-5156 (1991)], transient expression [Welch et al, Proc. Natl. Acad. Sci. USA. 88:10792-10796 (1991)], and infection [Preston et al, Virol. 186:87-98 (1992)] studies with the protease domain and the capsid assembly protein domain showed that the proteinase cleaves the capsid assembly protein near its carboxyl terminus. Further studies with the proteins produced in *E. coli* confirmed that the full-length protein of the UL26 ORF is capable of cleaving itself at two sites as well as cleaving the capsid assembly protein [Deckman et al, J. Virol. 66:7362-7367 (1992)]. DiIanni et al later located the cleavage sites between amino acids 247/248 and 610/611 of the UL26 ORF [J. Biol. Chem. 268:2048-2051 (1993)].

Although the results with ts1201 suggest that the defect in the virus is in its ability to cleave the capsid

assembly protein and subsequent encapsidation of DNA, it is not known whether this phenotype is the result of a defect in the protease activity *per se*, or whether the 5′ region of the UL26 ORF encodes some other functions required for capsid assembly and maturation. The processed proteinase domain of the 80 kDa precursor (designated as "VP24" or "N_O") has been identified in B-capsids [Davison et al, J. Gen. Virol. 73:2709-2713 (1992)] and is retained in A-capsids and C-capsids [F. J. Rixon, *Structure and Assembly of Herpesviruses*, in **Seminars in Virology**, vol. 4, 135-144, (A. J. Davison, ed. 1993)] suggesting a structural role for this domain. B-capsids are immature capsids in the nucleus of the infected cell that contain the capsid assembly protein, but not viral DNA. These capsids are thought to be the precursors of A-capsids which fail to package DNA and C-capsids which package DNA with concomitant loss of the capsid assembly protein [B. Roizman and A. Sears, *Herpes Simplex Viruses and Their Replication*, in **Human Herpesviruses**, 11-68, (B. Roizman, R. J. Whitley, and C. Lopez, eds. 1993)]. Gao et al constructed and characterized a null mutant virus ("m100") that contains a deletion within the protease domain of the HSV-1 UL26 gene [J. Virol. 68:3702-3712 (1994)]. The mutant virus could be propagated on a complementing cell line but not on noncomplementing Vero cells, indicating that the protease domain of UL26 is essential for viral replication in cell culture. DNA replication occurred at near wild-type levels, but the viral DNA was not processed to unit genome length or encapsidated.

We have generated a recombinant virus to further investigate the role of this domain with respect *in vivo* effects. The recombinant virus is avirulent *in vivo* and induces immunity to challenge by wild-type HSV-1.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the construction of four plasmids. (A) Plasmid pMON15839a contains a 3.4 kb *Kpn*I fragment of HSV-1 ("KOS") upstream of the SV40 polyadenylation signal. (B) Plasmid pMON15840 has a UL26 ORF downstream of the herpesvirus ICP6 promoter region. Translation of UL26 begins at methionine 10. (C) Sequence of the multiple cloning site inserted into the *Bsp*EI/*Bcl*I-digested pMON27005. (D) Plasmid pMON15835 contains an ICP6-β-glucuronidase cassette inserted into the *Bcl*I site of pMON27005. The UL26 ORF is shown as the stippled box, the ICP6 promoter region is shown as the hatched box. The plasmids are not drawn to scale. Abbreviations: "K", *Kpn*I; "B", *Bsg*I; "S", *Sma*I.

Figure 2 shows the Southern blot analysis of recombinant viruses. Viral DNA was digested with *Not*I or *Kpn*I, transferred to nitrocellulose and hybridized to an α-32P-dGTP-labeled 3.4 kb *Kpn*I fragment shown in Figure 1A. Schematics show the restriction maps of the viruses. The hatched region in the UL26 deletion schematic is the ICP6-β-glucuronidase insertion in the protease domain. Lane 1, HSV-1 ("17"); Lane 2, HSV/UL26/β-gluc; Lane 3, HSV/UL26/res. Abbreviations: "N", *Not*I; "K", *Kpn*I.

Figure 3 is a graphical representation which shows the multistep growth curves of mutant viruses. BHK/UL26 helper or BHK/C2 cells were infected at an MOI of 0.1. Cells were harvested at various time points and the virus was titered on BHK/UL26 helper cells. Circles represent virus from BHK/UL26 helper cells and squares represent virus from BHK/C2 cells. The error bars represent the ranges of duplicate determinations.

Figure 4 shows the capsid assembly protein processing results. BHK/C2 and BHK/UL26 helper cells were infected with an MOI of 5 for 18 hours. Cells were harvested and

proteins were separated on a 14% denaturing SDS-polyacrylamide gel, transferred to Immobilon membrane, and probed with antisera generated against a peptide of the HSV-1 capsid assembly protein (HSVAs-414). The open star indicates the major unprocessed assembly protein and the closed star indicates the processed form. Lane 1, mock infected cells; Lane 2, wild-type HSV-1; Lane 3, HSV/UL26/β-gluc; Lane 4, HSV/UL26/res.

Figure 5 is a graphical representation which shows the viral challenge of mice. Mice were inoculated intraperitoneal (i.p.) with 6 x 10^5 pfu of each virus and scored for mortality. The survivors and control mice (age- and sex-matched) were challenged with another dose of wild-type virus on day 32.

Figure 6 is a graphical representation which shows i.p. or subcutaneous (s.q.) inoculation of mice inoculated with media or 10^2, 10^4, or 10^6 pfu of HSV/UL26/β-gluc. Mice were inoculated on day 1 either i.p. (A) or s.q. (B). On day 31 the mice were challenged with 10^7 pfu of wild-type HSV-1 given i.p.

DESCRIPTION OF THE INVENTION

The present invention describes a vaccine comprising an assembly-deficient herpesvirus. Preferably, the herpesvirus contains an inactivated form of an essential protease gene. More preferably, the protease is required for the processing and assembly of immature, noninfectious capsid particles into mature, infectious capsid particles.

The protease gene can be inactivated by a method selected from deletion, insertion, substitution and any combination of deletion, insertion, or substitution. Preferably, the protease gene is inactivated by deletion of viral DNA and insertion or substitution of nonviral (heterologous) DNA. More preferably, the essential

protease gene is inactivated by deletion of viral DNA and insertion of nonviral (heterologous) DNA.

Preferably, the inactivated protease gene is selected from HSV-1 UL26, HSV-2 UL26, and HCMV UL80. More preferably, the protease is encoded by HSV-1 UL26.

The invention includes herpesviruses selected from HSV-1, HSV-2, HCMV, SCMV, VZV, EBV, HHV-6, HHV-7, HHV-8, PRV, BHV and EHV. Preferably, the virus is HSV-1 or HSV-2. More preferably, the virus is HSV-1. Preferably, the vaccine comprises the assembly-deficient mutant virus designated HSV/UL26/β-gluc.

Preferably, the vaccine comprises a dose between about 10 and about 10^6 plaque-forming units of said assembly-deficient herpesvirus.

Additionally, the present invention describes a method of manufacturing a vaccine comprising an assembly-deficient herpesvirus, by preparing stocks of the virus in a recombinant cell line capable of generating properly assembled virus. Preferably, the method of manufacturing a vaccine uses a virus selected from HSV-1, HSV-2, HCMV, SCMV, VZV, EBV, HHV-6, HHV-7, HHV-8, PRV, BHV, and EHV. More preferably, the method of manufacturing a vaccine uses virus selected from HSV-1 and HSV-2. Even more preferably, the method of manufacturing a vaccine uses a virus derived from HSV-1.

The present invention also describes a use of an assembly-deficient herpesvirus in a preparation of a vaccine.

Additionally, the present invention describes a method of immunizing a susceptible mammal against a herpesvirus by administering a vaccine comprising an assembly-deficient herpesvirus. Preferably, the susceptible mammal is selected from human, monkey, cow, horse, sheep, and pig. More preferably, the mammal is human.

The present invention also describes a mutant

herpesvirus containing an inactivated form of an essential protease gene required for the processing and assembly of immature, noninfectious capsid particles into mature, infectious capsid particles, said essential protease gene is inactivated by deletion of viral DNA and insertion of nonviral (heterologous) DNA.

Preferably, the essential protease gene is inactivated by deletion of a portion of the essential protease gene and insertion of a nonviral (heterologous) DNA segment comprising a reporter gene under the control of an inducible promoter. More preferably, the essential protease gene is the HSV-1 UL26 gene. More preferably, the inducible promoter is the HSV-1 ICP6 (UL39) promoter. Even more preferably, the nonviral (heterologus) DNA segment comprises the gusA gene encoding *E. coli* beta-glucuronidase under the control of an HSV-1 ICP6 (UL39) promoter.

The present invention additionally describes a recombinant host cell line expressing an essential protease gene under the control of an inducible promoter. Preferably, the recombinant host cell line is derived from a mammalian source. More preferably, the recombinant host cell line is derived from a rodent source. Even more preferably, the recombinant host cell line is BHK-21. Preferably, the inducible promoter is a herpesvirus promoter. More preferably, the inducible promoter is the HSV-1 ICP6 (UL39) promoter.

The present invention also describes a method of making mutant herpesviruses by introducing the virus into a recombinant host cell line and recovering mature viral particles harboring the mutant viral genome.

Definitions

The phrase "assembly-deficient" is intended to mean that the virus is able to replicate its DNA, but is unable to complete the steps of cleaving that DNA into genome-

length pieces and packaging that DNA into viral capsids.

The phrase "mature virion" is intended to mean a viral particle capable of infection in a susceptible host or cell type. The phrase "nonviral (heterologous) DNA" is intended to mean DNA that is not derived from a herpesvirus genome. The phrase "nonessential gene" is intended to mean a gene that can be disrupted by deletion, insertion, substitution, or a combination of deletion, substitution, and insertion of other DNA, and that a recombinant virus containing this disrupted gene can propagate in cultured cells that do not express nondisrupted copies of the same gene. The phrase "essential gene" is intended to mean a gene that is not a nonessential gene. The phrase "essential viral protease gene" is intended to mean an essential viral gene that encodes a protease.

EXPERIMENTAL

Baby hamster kidney cells (BHK-21) were obtained from American Type Culture Collection (ATCC) (Rockville, MD) and were cultured in Dulbecco's modified Eagle's media supplemented with 10% fetal bovine sera (JRH Biosciences, Lenexa, KS), 2 mM additional L-glutamine (JRH Biosciences) and 100 µg-units/ml of penicillin-streptomycin (JRH Biosciences). HSV-2 strain MS was obtained from ATCC and HSV-1 strain 17 was obtained from Dr. R. Lausch, University of South Alabama. Viral DNA was isolated and purified according to D'Aquila and Summers [J. Virol. 61:1291-1295 (1987)] for stock quantities and according to DeLuca et al [J. Virol. 52:767-776 (1984)] and Rader et al [J. Gen. Virol. 74:1858-1869 (1993)] for rapid Southern blot evaluation.

To generate cell lines that complement the defect in the UL26 gene, BHK-21 cells were cotransfected with 10 µg plasmid DNA containing the complementing sequences (see below) and 1 µg

SV2neo [P. J. Southern and P. Berg. J. Mol. Appl. Genet. 1:327-341 (1982)] using LipofectAmine Reagent (GIBCO/BRL/Life Technologies, Inc., Grand Island, NY) according to the manufacturer's instructions. After two days, cells were treated with trypsin and diluted into media containing 400 µg/ml G418 (Geneticin, Gibco/BRL/Life Technologies, Inc.). Individual colonies were isolated and expanded for determination of helper function.

Two plasmids were made for engineering a cell line that would complement a protease defective HSV-1. First, a 3.4 kb *Kpn*I fragment from HSV-1 (KOS) (from P. Olivo, Washington University) containing the entire UL26 promoter region and open reading frame (ORF) was subcloned into the *Kpn*I site of pMON3327 [Highkin et al, Poultry Science 70:970-981 (1991)] such that the SV40 polyadenylation signal is 3′ to the UL26 ORF. This plasmid was designated pMON15831a (Figure 1). The second plasmid consists of the UL26 ORF under control of the HSV-1 ICP6 (UL39) promoter region. This plasmid was synthesized in several steps. First, the 320 bp *Sma*I fragment containing the 5′ end of the UL26 ORF starting at nucleotide 18 was subcloned into the *Sma*I site of pUC18 resulting in pMON15838. The 1642 bp *Bsg*I-*Kpn*I fragment from pMON27010 was inserted into *Bsg*I-*Kpn*I digested pMON15838 to yield pMON15839. pMON27010 has the 3.4 kb *Kpn*I fragment from HSV-1 (strain 17) in pUC18. The 1956 bp *Eco*RI-*Hin*dIII fragment was isolated from pMON15839 and the ends were filled-in using Klenow polymerase before ligating to pMON15834 which had been digested with *Bam*HI and filled in as above. The resulting plasmid was designated pMON15840 (Figure 1). Plasmid pMON15834 has the filled-in 633 bp *Xho*I-*Sna*BI fragment of HSV-1 (strain 17) that directs the expression of the ICP6 ORF in the *Sma*I site of pMON3327.

A β-glucuronidase cassette was inserted into the UL26 ORF as follows: The β-glucuronidase cassette under control

of the HSV-1 ICP6 promoter region was constructed by isolating a 633 bp *Xho*I-*Sna*BI fragment from pMON27002. pMON27002 has the 16,191 bp *Sse*8387I D fragment from HSV-1 (strain 17) in pNEB193 (New England Biolabs, Beverly, MA). The *Xho*I site was filled-in using Klenow polymerase and was ligated into the filled-in *Nco*I site in pMON14327 (Luckow et al, J. Virol. 67:4566-4579 (1993)] which contains the β-glucuronidase gene. The new plasmid is designated pMON15833 (Figure 1). The *Not*I H fragment (6542 bp) containing the HSV-1 (strain 17) UL26 ORF was subcloned into *Not*I-digested pBS2SKP (Stratagene, La Jolla, CA) to generate plasmid pMON27005. pMON27005 was digested with *Bsp*EI and *Bcl*I. A polylinker containing multiple cloning sites and complementary ends was inserted to create plasmid pMON27026 (Figure 1). To construct a cassette for recombination with wild-type HSV-1 (strain 17), the 2871 bp ICP6-β-glucuronidase sequences were removed from pMON15833 by *Bam*HI digestion and ligated into *Bcl*I-digested pMON27026. The new vector is designated pMON15835 (Figure 1).

BHK cells were seeded at 4 x 10^5 cells per 60 mm dish one day prior to transfection. One microgram of genomic viral DNA and an equimolar amount of linearized plasmid containing the desired sequence changes were mixed with 25 µg of LipofectAmine in OptiMem media (Gibco/BRL/Life Technologies) and added to the cells for 4 hours. The media was aspirated and replaced by growth media. The transfected cells were completely lysed before the harvesting of the supernatant. Clarified, serially-diluted supernatant (0.8 ml) was plated onto the helper cell line in 60 mm dishes at 37 °C for 60 minutes. The inoculum was removed and the cells were overlaid with a 1% agarose (JRH Biosciences)/10% FBS/EMEM (BioWhitaker, Walkersville, MD). After the formation of visible cytopathic effects, 4 ml Dulbecco's phosphate-buffered saline (JRH Biosciences)

containing 300 μg/ml X-gluc (BioSynth AG, Switzerland) and 80 μg/mI neutral red (Sigma, St. Louis, MO) were added, and plaques were picked using a Pasteur pipette. For viruses containing the β-glucuronidase gene, blue plaques were selected. For rescued viruses (see below), clear plaques were selected. The viruses were plaque-purified three times or purified by limiting dilution. Purified virus was isolated and the DNA was analyzed by restriction enzyme analysis and Southern blotting [Maniatis et al, Molecular Cloning, A Laboratory Manual (1982)].

Analysis of the clear plaque virus in the blue plaque virus stock was done by the polymerase chain reaction (PCR) (Saiki et al, Science. 239:487-491 (1988)]. Two oligonucleotides that flanked the unique *Bsg*I site in the HSV-1 (strain 17) UL26 ORF were synthesized (Genosys, The Woodlands, TX). The forward primer was identical to nucleotides 50,913 to 50,932 of the HSV genome [5′-GGGCGAGTTGGCATTGGATC-3′, McGeoch et al, J. Gen. Virol. 69:1531-1574 (1988)]. The reverse primer was complementary to sequences 51,195 to 51,175 of the HSV-1 genome (5-AGACCGAGGGCAGGTAGTT-3′). Virus was extracted with phenol:chloroform and the viral DNA was ethanol-precipitated. The PCR was carried out using the GeneAmp PCR kit (Perkin-Elmer-Cetus, Norwalk, CT). The reaction products were analyzed on 5% polyacrylamide gels.

Peptide antibodies were raised in rabbits against regions corresponding to amino acids 414 through 428. Peptide HSVAs-414 (C-PAAGDPGVRGSGKR) was synthesized by Chiron Mimotopes Pty. Ltd. (Raleigh, NC) and purified to greater than 95% purity. HSVAs-414 mapped to the central region of the capsid assembly region of the UL26 and UL26.5 genes. The peptide had a free acid at the C-terminus and was conjugated to diphtheria toxoid at the N-terminus. Rabbits were inoculated with 100 μl of 1 μg/ml of protein mixed with an equal volume of Freund's complete adjuvant,

boosted with the same material in Freund's incomplete adjuvant at 4 week intervals beginning at week 2, and bled 10 and 17 days after boosting.

Cells were seeded in wells of six-well dishes at 5 x 10^5 cells/well. The next day, cells were infected with a multiplicity of infection (MOI) of 5 pfu/cell for 60 minutes at 37 °C with occasional gentle rocking. The inoculum was aspirated and growth media was added. At 18 hours post infection, the media was aspirated and 400 µl of 1X Protein Disruption Buffer (Novex, San Diego, CA) containing 10% β-mercaptoethanol were added. Proteins were separated on 14% Tris-glycine SDS-polyacrylamide gels (Novex) for 1.5 hours at 125 volts. The gels were incubated for 10 minutes in 1X Transfer Buffer (Novex) and blotted to Immobilon-P membranes (Novex) for 1-2 hours at 30 volts. The membranes were incubated in 1X Tris-buffered saline containing Tween 80 (TTBS), supplemented with 5% powdered milk for at least one hour (typically overnight). The blot was rinsed twice with TTBS for 15 minutes, and incubated with primary antibody for 1 hour at a dilution of 1/1000. The blot was rinsed twice with TTBS for 15 minutes before incubating with secondary antibody (alkaline phosphatase conjugated goat anti-rabbit antibody, Promega, Madison, WI) for 1 hour at a dilution of 1/4000. The alkaline phosphatase was visualized by incubating the blot in nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Promega) for 5 to 15 minutes, and the reaction stopped by rinsing extensively in H_2O.

Viral replication was examined by multistep growth analysis on the BHK/UL26 helper line and on BHK cells that did not contain the helper function but were G418-resistant (BHK/C2). Cells (1 x 10^{5}) were seeded in wells of a 24-well plate and infected with an MOI of 0.1 plaque-forming-units (pfu) per cell. At various times post infection, the infected cells were subjected to three rounds of freeze-

thawing [Tengelsen et al, J. Virol. 67:3470-3480 (1993)] and the lysates were titered on the BHK/UL26 helper line.

To generate cell lines capable of supporting replication recombinant viruses with a deletion and insertion within the UL26 open reading frame, BHK cells were cotransfected with pMON15831a which has the 3.4 kb *Kpn*I fragment of HSV-1 (KOS) 5′ to the SV40 polyadenylation signal (Figure 1) and SV2neo. G418-resistant cells were isolated and shown by Southern blot analysis to contain the HSV-1 *Kpn*I fragment. To determine which cell line would express the UL26 gene products, the cell lines were infected with HSV-2 (MS) to stimulate the UL26 promoter in the cell. HSV-1-specific anti-peptide antisera, generated by inoculating rabbits with the peptide HSVAs-414 conjugated to diphtheria toxin, was used to identify expression of the cellular UL26 gene products (data not shown). This cell line, designated BHK/UL26/8, was used for generation of recombinant viruses. A G418-resistant cell line which was cotransfected with pMON3327 and SV2neo serves as a control and is designated BHK/C2. An additional helper cell line (BHK/UL26 helper) was isolated after the discovery that significant amounts of rescued virus were being generated due to recombination with the *Kpn*I fragment present in BHK/UL26/8. This second line was transfected with plasmid pMON15840 which has the UL26 ORF behind the ICP6 promoter and lacks the large amount of HSV DNA 5′ to the UL26 ORF contained in pMON15831a. Translation from this integrated plasmid began at the methionine at the natural amino acid 10. Candidate cell lines were screened for their ability to support growth of the blue plaque phenotype recombinant virus (see below). A cell line isolated from this latter screening that supports the growth of the UL26 mutant virus was designated the BHK/UL26 helper cell line.

Cell line BHK/UL26/8 was transfected with HSV-1

(strain 17) genomic DNA and plasmid pMON15835 which contains a *Not*I fragment of HSV-1 (strain 17) with a deletion in the protease domain of the UL26 ORF and an insertion of the bacterial β-glucuronidase gene under control of the HSV-1 (strain 17) ICP6 promoter (Figure 1). After cell lysis, the supernatant was serially-diluted on BHK/UL26/8 and blue plaques were identified after 4 to 5 days post infection. The blue plaques were picked and plaque-purified three times. The recombinant virus was designated HSV/UL26/β-gluc. Plaque purification indicated poor segregation between the blue phenotype recombinant virus and a clear plaque phenotype virus which appeared to have a growth advantage, even on the helper cell line.

To determine the genotype and source of the clear plaque virus, DNA amplification was performed on cell-free viral DNA from the mixed culture of blue and clear plaque phenotype viruses. Amplification of a 283 bp fragment indicated the presence of wild-type virus in the stock. The PCR product was digested with *Bsg*I, which cuts the fragment from wild-type (strain 17) DNA, but does not cut the fragment from wild-type (strain KOS) DNA, which is the source of DNA in the helper cell (data not shown). Lack of digestion of the PCR product by *Bsg*I indicated that the wild-type virus was actually a revertant generated by recombination between the blue plaque phenotype virus and the UL26 sequences in the helper cell line. The rescued virus was designated HSV/UL26/res.

In order to generate a more pure stock of HSV/UL26/β-gluc, a new helper cell line (BHK/UL26 helper) was isolated in which the amount of HSV DNA sequence 5′ to the UL26 ORF was eliminated and replaced with the ICP6 promoter region fragment (pMON15840, Figure 1). Propagation of HSV/UL26/β-gluc on this cell line resulted in only the blue plaque phenotype.

Viral DNA from wild-type (strain 17), HSV/UL26/β-gluc

and the rescued virus was digested with *Not*I or *Kpn*I. The digested DNA was analyzed by Southern blot analysis after probing with a restriction fragment containing the full length UL26 open reading frame and 5′ flanking sequences. The results showed the expected pattern of digestion (Figure 2). Wild-type and rescued virus showed the same pattern as expected with both *Not*I (6.3 kb) and *Kpn*I (3.4 kb) digestion (Lanes 1 and 3). Deletion of a small region of the UL26 ORF and insertion of the β-glucuronidase gene resulted in addition of a new *Not*I site (resulting in predicted 4.8 and 4.4 kb fragments) and a new *Kpn*I site (resulting in a 4.0 and 2.1 kb fragments) (Lane 2) in HSV/UL26/β-gluc.

Growth curves were determined for the viruses on the different cell lines. At various times post infection, the cells were harvested and freeze-thawed three times before plating on BHK/UL26 helper cells. The results indicated that HSV/UL26/β-gluc failed to replicate in BHK/C2 cells but grew with wild-type kinetics on the BHK/UL26 helper cell line. The wild-type (strain 17) HSV-1 and the rescued virus replicated to identical titers and at identical rates on both BHK/C2 and the BHK/UL26 helper cell lines (Figure 3).

Since it has been shown by transient transfection experiments in mammalian cells, bacteria and ts1201 that certain mutations in the 5′ region of UL26 fail to cleave the capsid assembly protein [reviewed in Gao et al, J. Virol. 68:3702-3712 (1994)], HSV/UL26/β-gluc was used to infect BHK/C2, BHK and BHK/UL26 helper cells at an MOI of 5. At 18 hours post infection, the cells were lysed in SDS-PAGE sample buffer and proteins separated on a 14% SDS-PAGE gel. After transfer to Immobilon P membranes, the blots were incubated in antisera against the HSV-1 capsid assembly protein. The results are shown in Figure 4. Infection of BHK/C2 cells by HSV/UL26/β-gluc resulted in a

failure to process the capsid assembly protein to a lower molecular weight form. Infection of BHK/helper cells by HSV/UL26/β-gluc showed that the capsid assembly protein was appropriately processed. The rescued recombinant virus (HSV/UL26/res) processed the capsid assembly protein in both cell lines as did wild-type HSV-1 (lanes 2 and 4). The capsid assembly protein was made at normal levels during infection in both helper and non-helper cells but is not cleaved in the non-helper cells. The HSV/UL26/β-gluc recombinant fails to process the capsid assembly protein and has restricted growth.

Female Swiss-Webster mice (12-14 grams, Charles Rivers Laboratories, Wilmington, MA) were inoculated with virus intraperitoneally or subcutaneously with 100 μl volumes. Subcutaneous inoculations were delivered on the dorsal side near the base of the tail after brief CO_2/O_2 treatment of the mice. Virus was resuspended in DMEM containing 5% FBS unless otherwise noted. Food and water were given *ad libitum*. Mice were euthanized if they became moribund due to paralysis.

Mice were inoculated i.p. with 6 x 10^5 pfu (as determined on the helper cell line) of either the wild-type (strain 17) HSV, HSV/UL26/β-gluc, or the rescued virus in a 100 μl volume. As shown in Figure 5, mice infected with wild-type (strain 17) or the rescued virus died by day 7 post infection. All mice infected with HSV/UL26/β-gluc survived. The animals that originally received HSV/UL26/β-gluc were challenged with wild-type HSV-1 (strain 17), i.p., at the same dose given initially. Age- and sex-matched naive mice were also inoculated. One of the HSV/UL26/β-gluc infected mice was found dead about 16 hours post infection with the wild-type virus. Death was probably not related to the virus since it occurred so quickly after infection. The other 9 mice survived the wild-type virus challenge. The naive mice were susceptible

to wild-type virus infection although it took longer for the virus to cause morbidity and mortality (Figure 5).

In a second experiment, mice were inoculated i.p. with ten-fold serial dilutions of HSV/UL26/β-gluc starting at the same inoculum used in the initial experiment. On day 39, the mice were challenged i.p. with 6×10^6 pfu of HSV-1 (strain 17). This dose of wild-type virus was 10-fold higher than that in the initial experiment and resulted in 90% death in the mice that were initially inoculated with DMEM/5% FBS (Table 1, mock-infected set). Again, within 16 hours, 6 mice were found dead. Two of these were in the set that were previously inoculated with 10 pfu of HSV/UL26/β-gluc and 4 were in the set that were previously given 1×10^5 pfu of HSV/UL26/β-gluc. There was a significant difference among the six survival curves ($p<0.02$, log rank test). The data suggests that mice that were inoculated with HSV/UL26/β-gluc survived in a dose-dependent manner (Table 1). The survival curves of the mice receiving the highest dose of HSV/UL26/β-gluc were statistically different from the mock group ($p=0.023$, log rank test).

Table 1.

HSV/UL26/β-gluc	% Survival*
mock	10
6×10^1 pfu	12.5
6×10^2 pfu	30
6×10^3 pfu	60
6×10^4 pfu	50
6×10^5 pfu	83.3

* Survival determined on day 20 after i.p. challenge with 6×10^6 pfu of wild-type HSV-1 (strain 17). N = 10 for all groups except for the 6×10^1 (N = 8) and 6×10^5 (N = 6) due to the early death.

In a third experiment, virus stocks were prepared as previously but were resuspended in DMEM without any FBS. Sets of ten mice were inoculated with DMEM alone or with increasing doses of HSV/UL26/β-gluc by either i.p. or s.q. routes. After one month, all mice were challenged with 10^7 pfu of wild-type virus by i.p. inoculation. Some controls for rapid death included animals that received i.p. media then challenged with i.p. media, HSV/UL26/β-gluc and then media or, HSV/UL26/β-gluc and then challenged with HSV/UL26/β-gluc. None of these animals died during the course of the experiment. None of the experimental animals died within 24 hours of challenge. Of these, 90 animals had received two inoculations of virus and one would expect about 10-12% to have died rapidly. The results with the experimental groups are shown in Figure 6A and 6B. There was a significant difference among the survival curves for both the i.p. ($p<0.01$) and s.q. ($p<0.01$) inoculations (log rank test). Regression analysis shows that there is a dose-dependent effect of HSV/UL26/β-gluc on survival ($p<0.05$, Cochran-Armitage test) for both groups.

It is expected that this virus would have reduced efficiency and reactivate poorly, if at all. The fact that the mutation effects a late gene function suggests that the recombinant virus may be more efficacious in inducing immunity than viruses that have deletions in immediate early or early genes. The assembly-defective HSV/UL26/β-gluc virus is a member of a new class of vaccine candidates with a defect in late gene activity.

It is anticipated that the defect in the essential gene described in an assembly-deficient virus can be incorporated in a virus with other mutations in essential or nonessential genes. Such genes, like ICP47 of HSV-1, may modulate the host's ability to mount an immune reaction to the virus [Hill et al, Nature 375:411-415 (1995); Früh

et al, Nature 375:415-417 (1995)].

The vaccines of the present invention can be of a lyophilized form or suspended in a pharmaceutically-acceptable carrier. Suitable suspensions can include phosphate buffer, saline, glucose, inactivated serum, excipients, and adjuvants. The vaccine can be prepared and used according to standard techniques well known in the art [reviewed in R. L. Burke, Seminars in Virology, 4:187-197, (1993)]. The effective dose may also be determined by standard techniques well known in the art. Generally, vaccines are formulated in a suitable sterilized buffer and administered by intradermal, intramuscular, or subcutaneous injection at a dosage of between 10^3 and 10^9 pfu/kg. The vaccine can also be formulated for oral or ocular administration in vehicles known in the art.

The foregoing detailed description is given to facilitate clearness of understanding only, and no unnecessary limitations are to be understood therefrom, as modifications within the scope of the invention will be obvious to those skilled in the art.

What is claimed:

1. A vaccine comprising an assembly-deficient herpesvirus.

2. The vaccine of Claim 1 wherein said herpesvirus contains an inactivated form of an essential protease gene.

3. The vaccine of Claim 2 wherein said essential protease gene is required for the processing and assembly of immature, noninfectious capsid particles into mature, infectious capsid particles.

4. The vaccine of Claim 1 wherein said herpesvirus is selected from HSV-1, HSV-2, HCMV, SCMV, VZV, EBV, HHV-6, HHV-7, HHV-8, PRV, BHV and EHV.

5. The vaccine of Claim 4 wherein said herpesvirus is HSV-1 or HSV-2.

6. The vaccine of Claim 4 wherein said herpesvirus is HSV-1.

7. The vaccine of Claim 3 wherein said essential protease gene is selected from HSV-1 UL26, HSV-2 UL26, and HCMV UL80.

8. The vaccine of Claim 7 wherein said essential protease gene is HSV-1 UL26.

9. The vaccine of Claim 2 wherein said essential protease gene is inactivated by a method selected from deletion, insertion, substitution of DNA, and any combination of deletion, insertion, or substitution of DNA.

10. The vaccine of Claim 9 wherein said essential protease gene is inactivated by deletion of viral DNA and insertion of nonviral (heterologous) DNA.

11. The vaccine of Claim 1 comprising between about 10 and about 10^6 plaque-forming units of said herpesvirus.

12. The vaccine of Claim 1 wherein said assembly-deficient herpesvirus comprises the strain designated HSV/UL26/β-gluc.

13. A method of manufacturing a vaccine of Claim 1 comprising an assembly-deficient herpesvirus, by preparing stocks of said herpesvirus in a recombinant cell line capable of generating properly-assembled virus, and suspending said virus in a pharmaceutically-acceptable carrier.

14. The method of manufacturing a vaccine of Claim 13 wherein said essential protease gene is an HSV-1 UL26 gene.

15. The method of Claim 13 wherein said vaccine comprises the strain HSV/UL26/β-gluc.

16. The method of Claim 13 wherein said cell line is mammalian.

17. The method of Claim 16 wherein said cell line supports replication of said herpesvirus.

18. The method of Claim 17 wherein said cell line is the cell line designated BHK/UL26/8.

19. The method of Claim 17 wherein said cell line comprises the cell line designated BHK/UL26 helper.

20. A use of an assembly-deficient herpesvirus in a preparation of a vaccine.

21. A method of immunizing a mammal against a herpesvirus by administering a vaccine of Claim 1 in a pharmaceutically-acceptable carrier.

22. The method of Claim 21 where the mammal is selected from human, monkey, cow, horse, sheep and pig.

23. The method of Claim 22 where the mammal is human.

24. A mutant herpesvirus containing an inactivated form of an essential protease gene required for the processing and assembly of immature, noninfectious capsid particles into mature, infectious capsid particles, with said essential protease gene inactivated by deletion of viral DNA and insertion of nonviral (heterologous) DNA.

25. A mutant virus according to Claim 24 wherein said virus is selected from HSV-1, HSV-2, HCMV, SCMV, VZV, EBV, HHV-6, HHV-7, HHV-8, PRV, BHV and EHV.

26. A mutant virus of Claim 25 wherein said essential protease gene is HSV-1 UL26.

27. A mutant virus of Claim 24 wherein a portion of said essential protease gene is deleted and replaced by a nonviral (heterologous) DNA segment comprising a reporter gene under the control of an inducible herpesvirus HSV-1 promoter.

28. A mutant virus of Claim 27 wherein said reporter gene is selected from gusA encoding beta-glucuronidase, lacZ encoding beta-galactosidase, phoA encoding alkaline phosphatase, gfp encoding green fluorescent protein, and aeq encoding aequorin.

29. A mutant virus of Claim 28 wherein said reporter gene is the gusA gene encoding E. coli beta-glucuronidase.

30. A mutant virus of Claim 27 wherein said inducible herpesvirus promoter is the HSV-1 ICP6 (UL39) promoter.

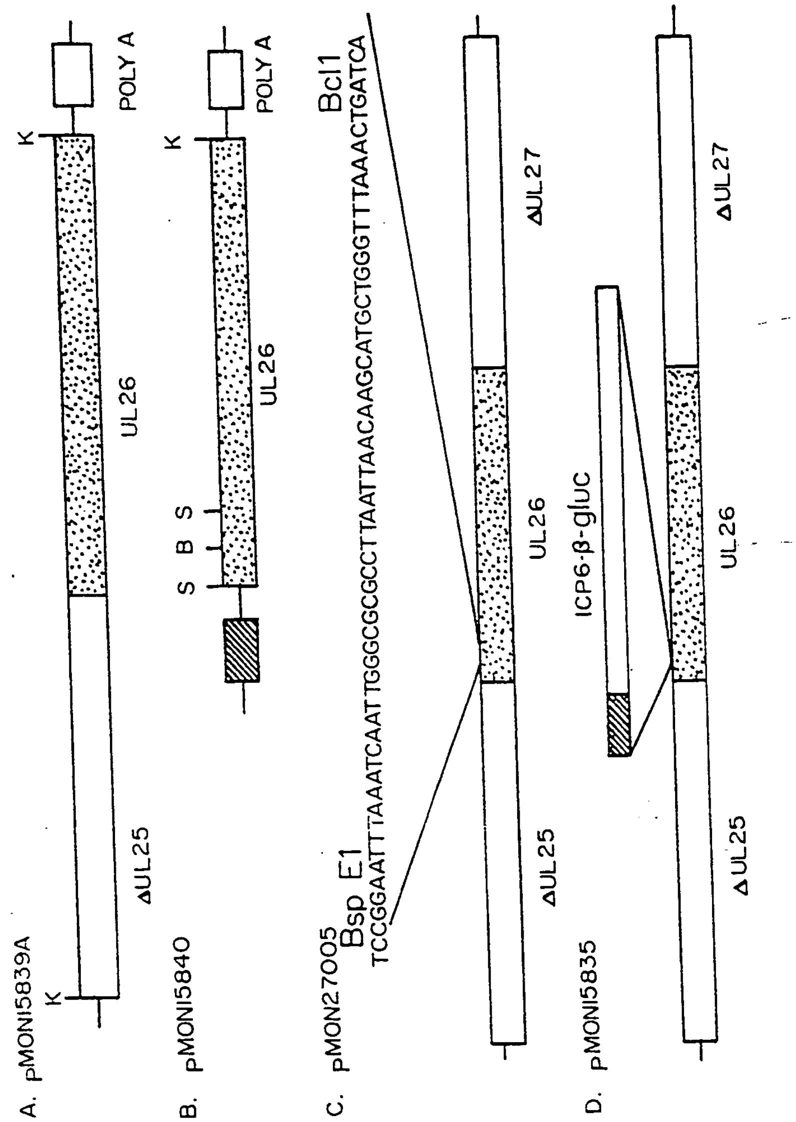
31. A recombinant host cell line expressing an essential herpesvirus protease gene under the control of an inducible non-protease promoter.

32. A recombinant host cell line of Claim 31, wherein said host cell line is from a rodent source.

33. A recombinant host cell line of Claim 32, wherein said host cell line is BHK-21.

34. A recombinant host cell line of Claim 31 wherein said inducible non-protease promoter is the HSV-1 ICP6 (UL39) promoter.

35. A method of making mutant herpesviruses of Claim 24 by introducing said virus into a recombinant host cell line and recovering mature viral particles harboring the mutant viral genome.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ΔUL25
UL26
K
POLY A
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S
B
S
UL26
K
POLY A
C. pMON27005
Bsp E1
TCCGGAATTTAAATCAATTGGCGCGCCTTAATTAACACAAGCATGCTGGTTTAAACTGATCA
Bcl1
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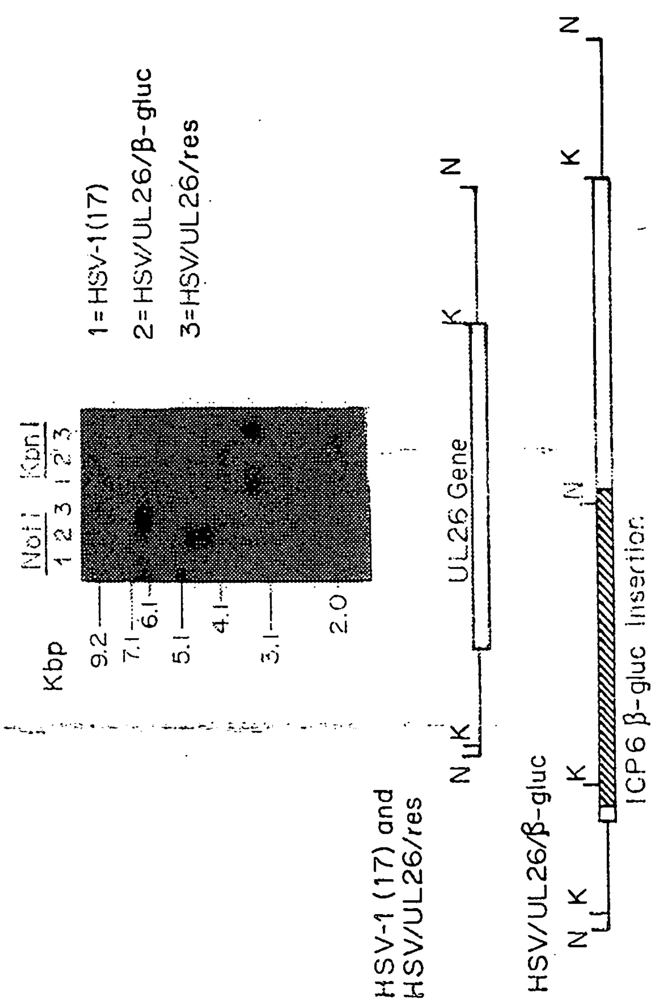
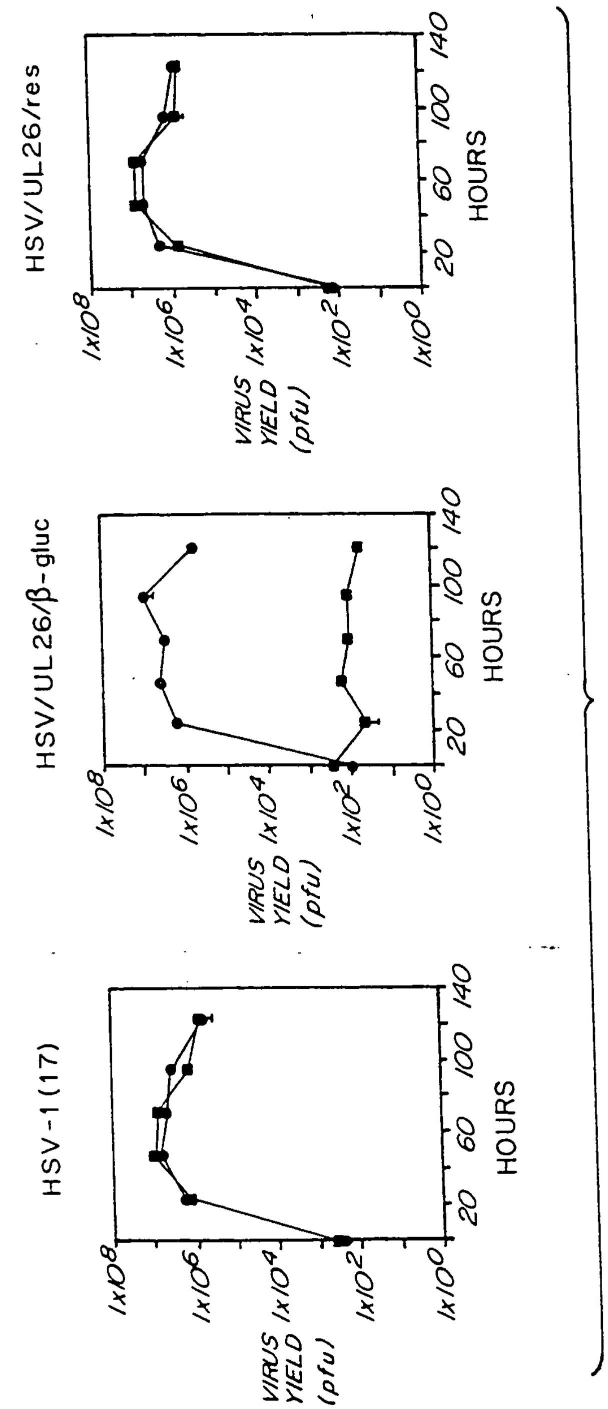
UL26
ΔUL27
D. pMON15835
ICP6-β-gluc
ΔUL25
UL26
ΔUL27

Fig. 1



Kbp
9.2
7.1
6.1
5.1
4.1
3.1
2.0
NotI
1 2 3
KpnI
1 2 3
1 = HSV-1(17)
2 = HSV/UL26/β-gluc
3 = HSV/UL26/res
UL26 Gene
HSV-1 (17) and
HSV/UL26/res
HSV/UL26/β-gluc
ICP6 β-gluc Insertion
N
K

Fig. 2


HSV-1 (17)
HSV/UL 26/β-gluc
HSV/UL26/res
VIRUS YIELD (pfu)
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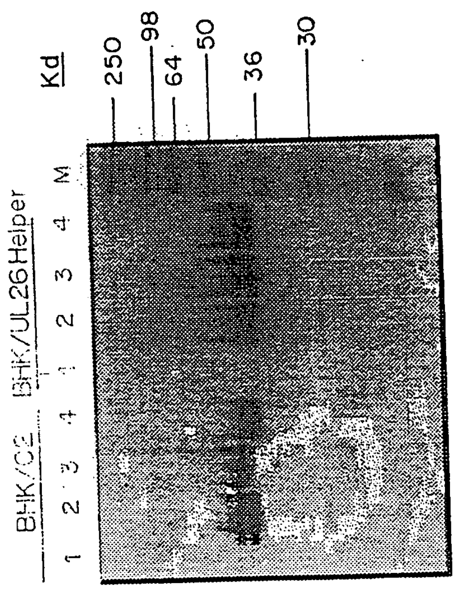
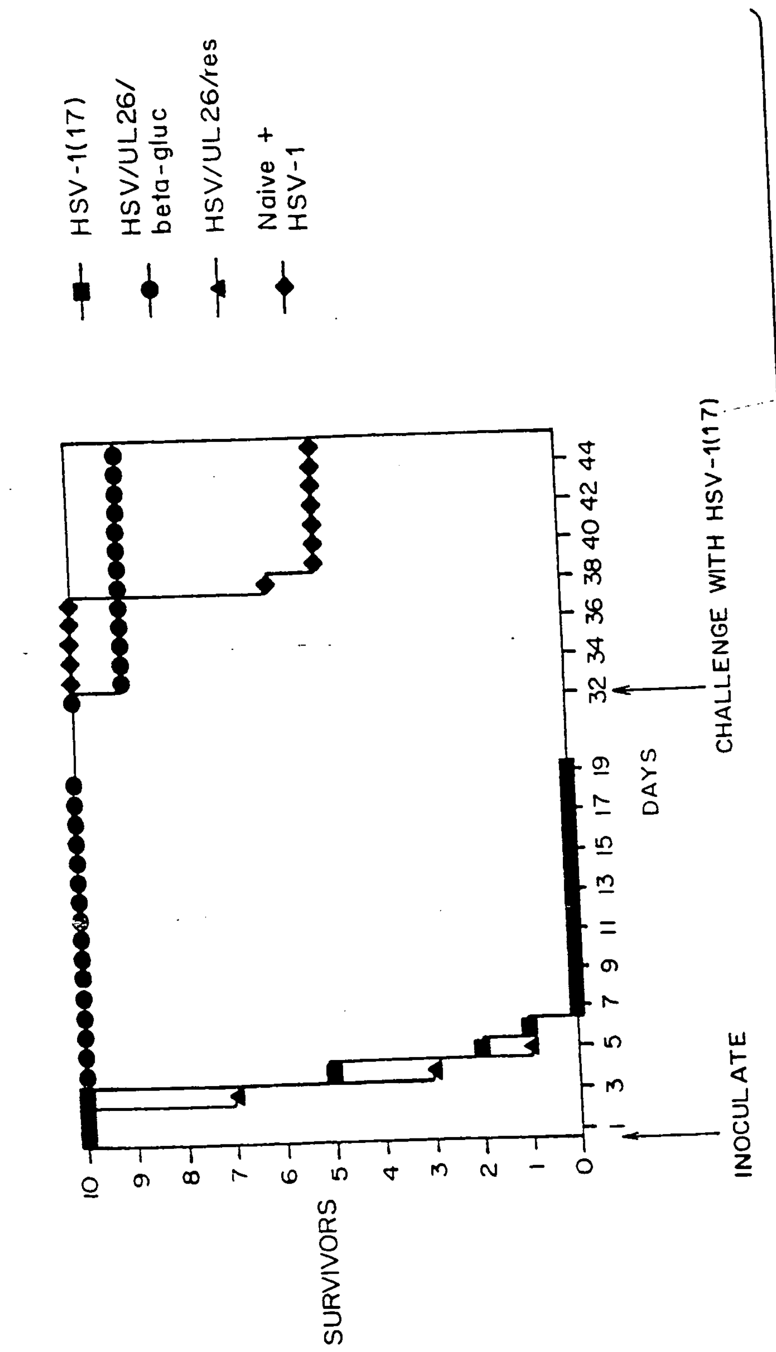
1x10^6
1x10^4
1x10^2
1x10^0
HOURS
20
60
100
140

Fig. 3



1 = Mock
2 = HSV-1 (17)
3 = HSV/UL26/β-gluc
4 = HSV/UL26/res
M = MW_r Markers

Fig. 4


HSV-1(17)
HSV/UL26/ beta-gluc
HSV/UL26/res
Naive + HSV-1
SURVIVORS
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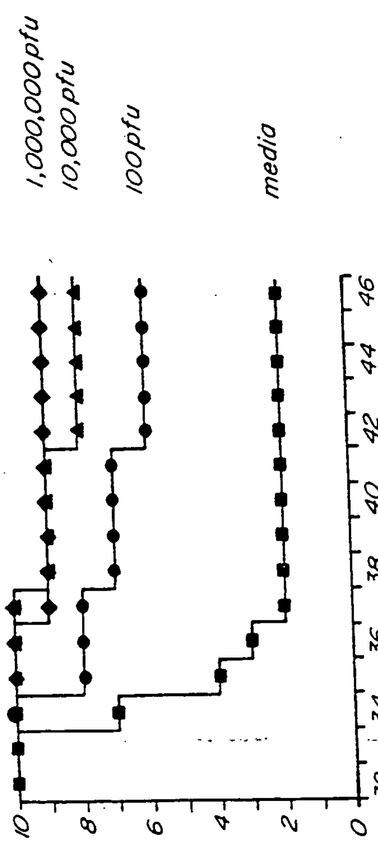
1 3 5 7 9 11 13 15 17 19 32 34 36 38 40 42 44
DAYS
INOCULATE
CHALLENGE WITH HSV-1(17)

Fig. 5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36
38
40
42
44
46
DAYS

Fig. 6A

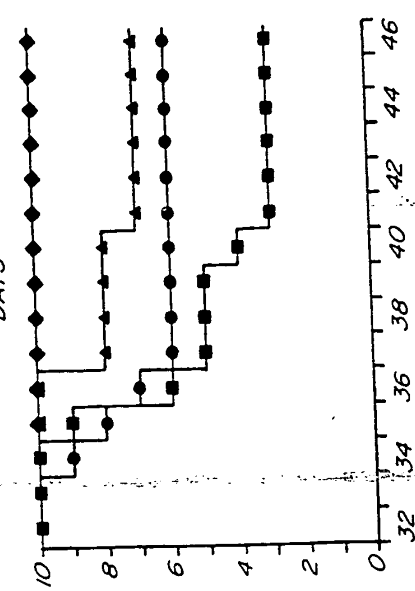
SURVIVORS
10
8
6
4
2
0
32
34
36
38
40
42
44
46
DAYS

Fig. 6B

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/14192

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/245 C12N15/52

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 96 38551 A (MERCK & CO. INC.) 5 December 1996 see page 1, line 18 - page 2, line 2 see page 6, line 1 - line 6 see page 8, line 18 - line 22 see page 8, line 29 - page 9, line 10 see page 23, line 18 - line 23	24-26, 31,35
X Y	WO 92 13943 A (SMITHKLINE BEECHAM BIOLOGICALS S.A.) 20 August 1992 see page 5, line 34 - page 6, line 7; claim 11; example 6	1-14,16, 17,20-23 15,18, 19,24-35

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
5 March 1998	27.03.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Olsen, L

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	M.F. AL-KOBAISI ET AL.: "The Herpes simplex virus UL33 gene product is required for the assembly of full capsids" VIROLOGY, vol. 180, 1991, pages 380-388, XP002057787 see the whole document ---	1-35
Y	D.R. THOMSEN ET AL.: "Assembly of the Herpes simplex virus capsid: requirement for the carboxyl-terminal twenty-five amino acids of the proteins encoded by the UL26 and UL26.5 genes" JOURNAL OF VIROLOGY, vol. 69, 1995, pages 3690-3703, XP002057788 see the whole document ---	1-35
X	WO 96 12007 A (MERCK & CO. INC.) 25 April 1996 see claim 4; example 1 -----	31

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 97/14192

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9638551 A	05-12-96	NONE	
WO 9213943 A	20-08-92	AU 657731 B	23-03-95
		AU 1182992 A	07-09-92
		CA 2101639 A	01-08-92
		EP 0571410 A	01-12-93
		JP 6507066 T	11-08-94
		NZ 241476 A	23-12-93
		NZ 245842 A	23-12-93
WO 9612007 A	25-04-96	AU 3952895 A	06-05-96

PCT

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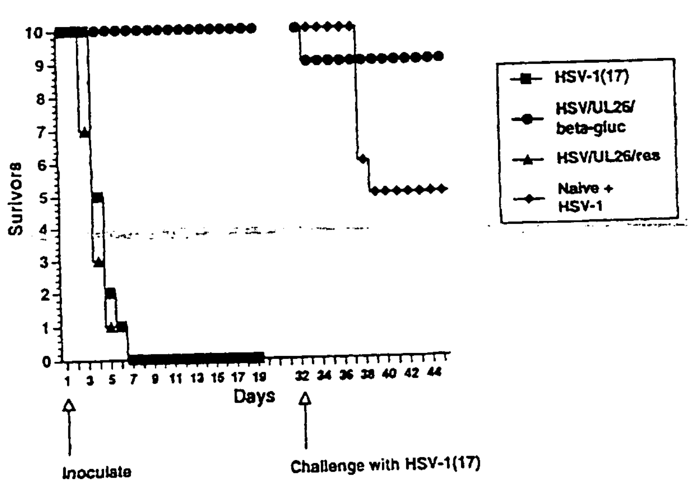

(57) Abstract

A vaccine is described which comprises an assembly-deficient herpesvirus. The mutant herpesvirus is capable of infecting and undergoing DNA replication in the cells of a susceptible mammal, but is defective in capsid assembly and formation of mature virion particles. The assembly-deficient herpesvirus is avirulent and capable of generating a protective immune response in a vaccinated mammal.

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ASSEMBLY-DEFICIENT HERPESVIRUS VACCINE

FIELD OF THE INVENTION

This invention is in the field of viral vaccines, and specifically relates to the generation of assembly-deficient mutant herpesviruses, vaccines comprising assembly-deficient mutant herpesviruses, and methods for the production and manufacture of assembly-deficient herpesvirus vaccines.

BACKGROUND OF THE INVENTION

There is a great need for therapies for the treatment of viral diseases. While antiviral drugs such as zidovudine, used in the treatment of human immunodeficiency virus (HIV), and drugs such as ganciclovir, acyclovir, and foscarnet are used in the treatment of herpesvirus infections, significant side effects often limit their effectiveness. The selection and spread of drug-resistant viruses also limits the effectiveness of small molecular weight antiviral drugs. This is a particularly significant problem for drugs targeted against RNA viruses such as HIV, which have a relatively high mutation rate compared to most DNA viruses.

Antiviral vaccines are a viable alternative to postinfection antiviral drug treatments. Ideally, antiviral vaccines protect against primary disease and recurring infections. Efficacy against a particular disease is crucial to the development of a vaccine strategy. Regulatory concerns, particularly related to the safety of vaccines intended for prophylactic use in healthy individuals, must also be considered.

While herpesvirus vaccines have been an active area of both academic and commercial interest, induction of a good,

protective immune response in humans has been challenging [R. L. Burke, *Current Status of HSV Vaccine Development*, in **The Human Herpesviruses**, 367-379, (B. Roizman, R. J. Whitley and C. Lopez, eds. 1993)]. Live virus vaccines have the risk of establishing latency and reactivating. Live virus vaccines also have the potential of recombining with natural isolates.

Attenuated recombinant viruses and subunit vaccines have been investigated to avoid these risks. Meignier et al describe a recombinant virus resulting from the removal of a region of herpes simplex virus type 1 (HSV-1) required for virulence and the insertion of herpes simplex virus type 2 (HSV-2) glycoprotein genes [J. Infect. Dis., 158:602-614 (1988)]. The viruses had reduced pathogenicity and induced immunity in a number of animal models.

More recently, recombinant herpes simplex viruses with deletions in essential immediate early or early genes have been described. These recombinant viruses are described as being efficacious in inducing immunity and reducing acute replication and establishment of latency of the challenged wild-type virus in mice. Nguyen et al describe replication-defective mutants of HSV-1 that have mutations in the essential genes encoding infected cell protein 8 ("ICP8") or ICP27 [J. Virol. 66:7067-7072 (1992)]. The ICP8 mutant (d301) expresses the products of the α and β genes while the ICP27 mutant (n504) expresses the products of the α, β, and γ_1 genes in the cells that the viruses can infect. Both viruses induced antibody responses that were lower than parental (KOS 1.1) virus, but the level induced by the ICP27 mutant was higher than that induced by infection with the ICP8 mutant. Morrison and Knipe later demonstrated that injection of these viruses protected mice against development of encephalitis and keratitis, and decreased the primary replication of virulent challenge

virus [J. Virol. 68:689-696 (1994)]. WO95/18852 describes similar replication-defective herpesvirus mutants and WO94/03207 describes vaccines based on these mutants.

Another recombinant virus has been described that has a deletion in the glycoprotein H (gH) coding region [Forrester et al, J. Virol. 66:341-348 (1992); WO92/05263]. This virus forms virions after infection of non-helper cells, but the viruses fail to infect in a subsequent round. Inoculation of mice with the gH deletion virus resulted in a more rapid clearance of the wild-type challenge virus compared to vaccination with chemically-inactivated virus [Farrell et al, J. Virol. 68:927-932 (1994)]. Inoculation of guinea pigs with the gH deleted recombinant virus resulted in reduced primary vaginal disease and reduced recurrences [McLean et al, J. Infect. Dis. 170:1100-1109 (1994)].

Most viruses encode proteinases that function in the processing of viral proteins during infection [W. G. Dougherty and B. L. Semler, Microbiological Reviews, 57:781-822 (1993)]. Biological and biochemical studies have shown that HSV-1 possesses a proteinase that can process another viral protein, the capsid assembly protein (also known as p40, ICP35 and VP22a). Similar proteinases are encoded in the genome of other members of the Herpesviridae. This family of DNA viruses includes HSV-1, HSV-2, human and simian cytomegalovirus (HCMV, SCMV), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), human herpesvirus types -6, -7, and -8 (HHV-6, HHV-7, and HHV-8), pseudorabies virus (PRV), bovine herpesvirus (BHV), equine herpesvirus (EHV), and rhinotracheitis virus, among others.

Early work by Preston et al, [J. Virol. 45:1056-1064 (1983)] showed that a temperature-sensitive (ts) mutant in HSV-1 (ts1201) failed to cleave the capsid assembly protein to its lower molecular weight forms at the nonpermissive

temperature. This mutant also failed to package viral DNA. By marker rescue, the defect was mapped to a region of the genome in what is now known as the UL26 open reading frame (ORF) [McGeoch et al, J. Gen. Virol. 69:1531-1574 (1988)]. Subsequent analysis showed that two transcripts initiate in the UL26 region, a primary transcript of about 2.1 kb which encodes a protein of 635 amino acids, and a more abundant transcript which is initiated within the UL26 ORF, about 1000 nucleotides 3′ of the primary transcript initiation. This smaller transcript encodes a predicted protein of 329 amino acids and is 3′ coterminal with the larger 80 kDa ORF encoded by the larger transcript [F. Y. Liu and B. Roizman. J. Virol. 65:206-212 (1991)]. The defect in the ts1201 mutant maps in the 5′ region of the longer transcript which has been shown to encode a proteinase activity in HSV-1 [F. Y. Liu and B. Roizman. J. Virol. 65:5149-5156 (1991)] or in simian cytomegalovirus [Welch et al, Proc. Natl. Acad. Sci. USA. 88:10792-10796 (1991)].

Superinfection/transient expression [F. Y. Liu and B. Roizman. J. Virol. 65:5149-5156 (1991)], transient expression [Welch et al, Proc. Natl. Acad. Sci. USA. 88:10792-10796 (1991)], and infection [Preston et al, Virol. 186:87-98 (1992)] studies with the protease domain and the capsid assembly protein domain showed that the proteinase cleaves the capsid assembly protein near its carboxyl terminus. Further studies with the proteins produced in *E. coli* confirmed that the full-length protein of the UL26 ORF is capable of cleaving itself at two sites as well as cleaving the capsid assembly protein [Deckman et al, J. Virol. 66:7362-7367 (1992)]. DiIanni et al later located the cleavage sites between amino acids 247/248 and 610/611 of the UL26 ORF [J. Biol. Chem. 268:2048-2051 (1993)].

Although the results with ts1201 suggest that the defect in the virus is in its ability to cleave the capsid

assembly protein and subsequent encapsidation of DNA, it is not known whether this phenotype is the result of a defect in the protease activity *per se*, or whether the 5′ region of the UL26 ORF encodes some other functions required for capsid assembly and maturation. The processed proteinase domain of the 80 kDa precursor (designated as "VP24" or "N_O") has been identified in B-capsids [Davison et al, J. Gen. Virol. 73:2709-2713 (1992)] and is retained in A-capsids and C-capsids [F. J. Rixon, *Structure and Assembly of Herpesviruses*, in **Seminars in Virology**, vol. 4, 135-144, (A. J. Davison, ed. 1993)] suggesting a structural role for this domain. B-capsids are immature capsids in the nucleus of the infected cell that contain the capsid assembly protein, but not viral DNA. These capsids are thought to be the precursors of A-capsids which fail to package DNA and C-capsids which package DNA with concomitant loss of the capsid assembly protein [B. Roizman and A. Sears, *Herpes Simplex Viruses and Their Replication*, in **Human Herpesviruses**, 11-68, (B. Roizman, R. J. Whitley, and C. Lopez, eds. 1993)]. Gao et al constructed and characterized a null mutant virus ("m100") that contains a deletion within the protease domain of the HSV-1 UL26 gene [J. Virol. 68:3702-3712 (1994)]. The mutant virus could be propagated on a complementing cell line but not on noncomplementing Vero cells, indicating that the protease domain of UL26 is essential for viral replication in cell culture. DNA replication occurred at near wild-type levels, but the viral DNA was not processed to unit genome length or encapsidated.

We have generated a recombinant virus to further investigate the role of this domain with respect *in vivo* effects. The recombinant virus is avirulent *in vivo* and induces immunity to challenge by wild-type HSV-1.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the construction of four plasmids. (A) Plasmid pMON15839a contains a 3.4 kb *Kpn*I fragment of HSV-1 ("KOS") upstream of the SV40 polyadenylation signal. (B) Plasmid pMON15840 has a UL26 ORF downstream of the herpesvirus ICP6 promoter region. Translation of UL26 begins at methionine 10. (C) Sequence of the multiple cloning site inserted into the *Bsp*EI/*Bcl*I-digested pMON27005. (D) Plasmid pMON15835 contains an ICP6-β-glucuronidase cassette inserted into the *Bcl*I site of pMON27005. The UL26 ORF is shown as the stippled box, the ICP6 promoter region is shown as the hatched box. The plasmids are not drawn to scale. Abbreviations: "K", *Kpn*I; "B", *Bsg*I; "S", *Sma*I.

Figure 2 shows the Southern blot analysis of recombinant viruses. Viral DNA was digested with *Not*I or *Kpn*I, transferred to nitrocellulose and hybridized to an α-32P-dGTP-labeled 3.4 kb *Kpn*I fragment shown in Figure 1A. Schematics show the restriction maps of the viruses. The hatched region in the UL26 deletion schematic is the ICP6-β-glucuronidase insertion in the protease domain. Lane 1, HSV-1 ("17"); Lane 2, HSV/UL26/β-gluc; Lane 3, HSV/UL26/res. Abbreviations: "N", *Not*I; "K", *Kpn*I.

Figure 3 is a graphical representation which shows the multistep growth curves of mutant viruses. BHK/UL26 helper or BHK/C2 cells were infected at an MOI of 0.1. Cells were harvested at various time points and the virus was titered on BHK/UL26 helper cells. Circles represent virus from BHK/UL26 helper cells and squares represent virus from BHK/C2 cells. The error bars represent the ranges of duplicate determinations.

Figure 4 shows the capsid assembly protein processing results. BHK/C2 and BHK/UL26 helper cells were infected with an MOI of 5 for 18 hours. Cells were harvested and

proteins were separated on a 14% denaturing SDS-polyacrylamide gel, transferred to Immobilon membrane, and probed with antisera generated against a peptide of the HSV-1 capsid assembly protein (HSVAs-414). The open star indicates the major unprocessed assembly protein and the closed star indicates the processed form. Lane 1, mock infected cells; Lane 2, wild-type HSV-1; Lane 3, HSV/UL26/β-gluc; Lane 4, HSV/UL26/res.

Figure 5 is a graphical representation which shows the viral challenge of mice. Mice were inoculated intraperitoneal (i.p.) with 6 x 10^5 pfu of each virus and scored for mortality. The survivors and control mice (age- and sex-matched) were challenged with another dose of wild-type virus on day 32.

Figure 6 is a graphical representation which shows i.p. or subcutaneous (s.q.) inoculation of mice inoculated with media or 10^2, 10^4, or 10^6 pfu of HSV/UL26/β-gluc. Mice were inoculated on day 1 either i.p. (A) or s.q. (B). On day 31 the mice were challenged with 10^7 pfu of wild-type HSV-1 given i.p.

DESCRIPTION OF THE INVENTION

The present invention describes a vaccine comprising an assembly-deficient herpesvirus. Preferably, the herpesvirus contains an inactivated form of an essential protease gene. More preferably, the protease is required for the processing and assembly of immature, noninfectious capsid particles into mature, infectious capsid particles.

The protease gene can be inactivated by a method selected from deletion, insertion, substitution and any combination of deletion, insertion, or substitution. Preferably, the protease gene is inactivated by deletion of viral DNA and insertion or substitution of nonviral (heterologous) DNA. More preferably, the essential

protease gene is inactivated by deletion of viral DNA and insertion of nonviral (heterologous) DNA.

Preferably, the inactivated protease gene is selected from HSV-1 UL26, HSV-2 UL26, and HCMV UL80. More preferably, the protease is encoded by HSV-1 UL26.

The invention includes herpesviruses selected from HSV-1, HSV-2, HCMV, SCMV, VZV, EBV, HHV-6, HHV-7, HHV-8, PRV, BHV and EHV. Preferably, the virus is HSV-1 or HSV-2. More preferably, the virus is HSV-1. Preferably, the vaccine comprises the assembly-deficient mutant virus designated HSV/UL26/β-gluc.

Preferably, the vaccine comprises a dose between about 10 and about 10^6 plaque-forming units of said assembly-deficient herpesvirus.

Additionally, the present invention describes a method of manufacturing a vaccine comprising an assembly-deficient herpesvirus, by preparing stocks of the virus in a recombinant cell line capable of generating properly assembled virus. Preferably, the method of manufacturing a vaccine uses a virus selected from HSV-1, HSV-2, HCMV, SCMV, VZV, EBV, HHV-6, HHV-7, HHV-8, PRV, BHV, and EHV. More preferably, the method of manufacturing a vaccine uses virus selected from HSV-1 and HSV-2. Even more preferably, the method of manufacturing a vaccine uses a virus derived from HSV-1.

The present invention also describes a use of an assembly-deficient herpesvirus in a preparation of a vaccine.

Additionally, the present invention describes a method of immunizing a susceptible mammal against a herpesvirus by administering a vaccine comprising an assembly-deficient herpesvirus. Preferably, the susceptible mammal is selected from human, monkey, cow, horse, sheep, and pig. More preferably, the mammal is human.

The present invention also describes a mutant

herpesvirus containing an inactivated form of an essential protease gene required for the processing and assembly of immature, noninfectious capsid particles into mature, infectious capsid particles, said essential protease gene is inactivated by deletion of viral DNA and insertion of nonviral (heterologous) DNA.

Preferably, the essential protease gene is inactivated by deletion of a portion of the essential protease gene and insertion of a nonviral (heterologous) DNA segment comprising a reporter gene under the control of an inducible promoter. More preferably, the essential protease gene is the HSV-1 UL26 gene. More preferably, the inducible promoter is the HSV-1 ICP6 (UL39) promoter. Even more preferably, the nonviral (heterologus) DNA segment comprises the gusA gene encoding E. coli beta-glucuronidase under the control of an HSV-1 ICP6 (UL39) promoter.

The present invention additionally describes a recombinant host cell line expressing an essential protease gene under the control of an inducible promoter. Preferably, the recombinant host cell line is derived from a mammalian source. More preferably, the recombinant host cell line is derived from a rodent source. Even more preferably, the recombinant host cell line is BHK-21. Preferably, the inducible promoter is a herpesvirus promoter. More preferably, the inducible promoter is the HSV-1 ICP6 (UL39) promoter.

The present invention also describes a method of making mutant herpesviruses by introducing the virus into a recombinant host cell line and recovering mature viral particles harboring the mutant viral genome.

Definitions

The phrase "assembly-deficient" is intended to mean that the virus is able to replicate its DNA, but is unable to complete the steps of cleaving that DNA into genome-

length pieces and packaging that DNA into viral capsids.

The phrase "mature virion" is intended to mean a viral particle capable of infection in a susceptible host or cell type. The phrase "nonviral (heterologous) DNA" is intended to mean DNA that is not derived from a herpesvirus genome. The phrase "nonessential gene" is intended to mean a gene that can be disrupted by deletion, insertion, substitution, or a combination of deletion, substitution, and insertion of other DNA, and that a recombinant virus containing this disrupted gene can propagate in cultured cells that do not express nondisrupted copies of the same gene. The phrase "essential gene" is intended to mean a gene that is not a nonessential gene. The phrase "essential viral protease gene" is intended to mean an essential viral gene that encodes a protease.

EXPERIMENTAL

Baby hamster kidney cells (BHK-21) were obtained from American Type Culture Collection (ATCC) (Rockville, MD) and were cultured in Dulbecco's modified Eagle's media supplemented with 10% fetal bovine sera (JRH Biosciences, Lenexa, KS), 2 mM additional L-glutamine (JRH Biosciences) and 100 µg-units/ml of penicillin-streptomycin (JRH Biosciences). HSV-2 strain MS was obtained from ATCC and HSV-1 strain 17 was obtained from Dr. R. Lausch, University of South Alabama. Viral DNA was isolated and purified according to D'Aquila and Summers [J. Virol. 61:1291-1295 (1987)] for stock quantities and according to DeLuca et al [J. Virol. 52:767-776 (1984)] and Rader et al [J. Gen. Virol. 74:1858-1869 (1993)] for rapid Southern blot evaluation.

To generate cell lines that complement the defect in the UL26 gene, BHK-21 cells were cotransfected with 10 µg plasmid DNA containing the complementing sequences (see below) and 1 µg

SV2neo [P. J. Southern and P. Berg. J. Mol. Appl. Genet. 1:327-341 (1982)] using LipofectAmine Reagent (GIBCO/BRL/Life Technologies, Inc., Grand Island, NY) according to the manufacturer's instructions. After two days, cells were treated with trypsin and diluted into media containing 400 µg/ml G418 (Geneticin, Gibco/BRL/Life Technologies, Inc.). Individual colonies were isolated and expanded for determination of helper function.

Two plasmids were made for engineering a cell line that would complement a protease defective HSV-1. First, a 3.4 kb *Kpn*I fragment from HSV-1 (KOS) (from P. Olivo, Washington University) containing the entire UL26 promoter region and open reading frame (ORF) was subcloned into the *Kpn*I site of pMON3327 [Highkin et al, Poultry Science 70:970-981 (1991)] such that the SV40 polyadenylation signal is 3′ to the UL26 ORF. This plasmid was designated pMON15831a (Figure 1). The second plasmid consists of the UL26 ORF under control of the HSV-1 ICP6 (UL39) promoter region. This plasmid was synthesized in several steps. First, the 320 bp *Sma*I fragment containing the 5′ end of the UL26 ORF starting at nucleotide 18 was subcloned into the *Sma*I site of pUC18 resulting in pMON15838. The 1642 bp *Bsg*I-*Kpn*I fragment from pMON27010 was inserted into *Bsg*I-*Kpn*I digested pMON15838 to yield pMON15839. pMON27010 has the 3.4 kb *Kpn*I fragment from HSV-1 (strain 17) in pUC18. The 1956 bp *Eco*RI-*Hin*dIII fragment was isolated from pMON15839 and the ends were filled-in using Klenow polymerase before ligating to pMON15834 which had been digested with *Bam*HI and filled in as above. The resulting plasmid was designated pMON15840 (Figure 1). Plasmid pMON15834 has the filled-in 633 bp *Xho*I-*Sna*BI fragment of HSV-1 (strain 17) that directs the expression of the ICP6 ORF in the *Sma*I site of pMON3327.

A β-glucuronidase cassette was inserted into the UL26 ORF as follows: The β-glucuronidase cassette under control

of the HSV-1 ICP6 promoter region was constructed by isolating a 633 bp *Xho*I-*Sna*BI fragment from pMON27002. pMON27002 has the 16,191 bp *Sse*8387I D fragment from HSV-1 (strain 17) in pNEB193 (New England Biolabs, Beverly, MA). The *Xho*I site was filled-in using Klenow polymerase and was ligated into the filled-in *Nco*I site in pMON14327 (Luckow et al, J. Virol. 67:4566-4579 (1993)] which contains the β-glucuronidase gene. The new plasmid is designated pMON15833 (Figure 1). The *Not*I H fragment (6542 bp) containing the HSV-1 (strain 17) UL26 ORF was subcloned into *Not*I-digested pBS2SKP (Stratagene, La Jolla, CA) to generate plasmid pMON27005. pMON27005 was digested with *Bsp*EI and *Bcl*I. A polylinker containing multiple cloning sites and complementary ends was inserted to create plasmid pMON27026 (Figure 1). To construct a cassette for recombination with wild-type HSV-1 (strain 17), the 2871 bp ICP6-β-glucuronidase sequences were removed from pMON15833 by *Bam*HI digestion and ligated into *Bcl*I-digested pMON27026. The new vector is designated pMON15835 (Figure 1).

BHK cells were seeded at 4 x 10^5 cells per 60 mm dish one day prior to transfection. One microgram of genomic viral DNA and an equimolar amount of linearized plasmid containing the desired sequence changes were mixed with 25 µg of LipofectAmine in OptiMem media (Gibco/BRL/Life Technologies) and added to the cells for 4 hours. The media was aspirated and replaced by growth media. The transfected cells were completely lysed before the harvesting of the supernatant. Clarified, serially-diluted supernatant (0.8 ml) was plated onto the helper cell line in 60 mm dishes at 37 °C for 60 minutes. The inoculum was removed and the cells were overlaid with a 1% agarose (JRH Biosciences)/10% FBS/EMEM (BioWhitaker, Walkersville, MD). After the formation of visible cytopathic effects, 4 ml Dulbecco's phosphate-buffered saline (JRH Biosciences)

containing 300 μg/ml X-gluc (BioSynth AG, Switzerland) and 80 μg/mI neutral red (Sigma, St. Louis, MO) were added, and plaques were picked using a Pasteur pipette. For viruses containing the β-glucuronidase gene, blue plaques were selected. For rescued viruses (see below), clear plaques were selected. The viruses were plaque-purified three times or purified by limiting dilution. Purified virus was isolated and the DNA was analyzed by restriction enzyme analysis and Southern blotting [Maniatis et al, Molecular Cloning, A Laboratory Manual (1982)].

Analysis of the clear plaque virus in the blue plaque virus stock was done by the polymerase chain reaction (PCR) (Saiki et al, Science. 239:487-491 (1988)]. Two oligonucleotides that flanked the unique *Bsg*I site in the HSV-1 (strain 17) UL26 ORF were synthesized (Genosys, The Woodlands, TX). The forward primer was identical to nucleotides 50,913 to 50,932 of the HSV genome [5′-GGGCGAGTTGGCATTGGATC-3′, McGeoch et al, J. Gen. Virol. 69:1531-1574 (1988)]. The reverse primer was complementary to sequences 51,195 to 51,175 of the HSV-1 genome (5-AGACCGAGGGCAGGTAGTT-3′). Virus was extracted with phenol:chloroform and the viral DNA was ethanol-precipitated. The PCR was carried out using the GeneAmp PCR kit (Perkin-Elmer-Cetus, Norwalk, CT). The reaction products were analyzed on 5% polyacrylamide gels.

Peptide antibodies were raised in rabbits against regions corresponding to amino acids 414 through 428. Peptide HSVAs-414 (C-PAAGDPGVRGSGKR) was synthesized by Chiron Mimotopes Pty. Ltd. (Raleigh, NC) and purified to greater than 95% purity. HSVAs-414 mapped to the central region of the capsid assembly region of the UL26 and UL26.5 genes. The peptide had a free acid at the C-terminus and was conjugated to diphtheria toxoid at the N-terminus. Rabbits were inoculated with 100 μl of 1 μg/ml of protein mixed with an equal volume of Freund's complete adjuvant,

boosted with the same material in Freund's incomplete adjuvant at 4 week intervals beginning at week 2, and bled 10 and 17 days after boosting.

Cells were seeded in wells of six-well dishes at 5 x 10^5 cells/well. The next day, cells were infected with a multiplicity of infection (MOI) of 5 pfu/cell for 60 minutes at 37 °C with occasional gentle rocking. The inoculum was aspirated and growth media was added. At 18 hours post infection, the media was aspirated and 400 µl of 1X Protein Disruption Buffer (Novex, San Diego, CA) containing 10% β-mercaptoethanol were added. Proteins were separated on 14% Tris-glycine SDS-polyacrylamide gels (Novex) for 1.5 hours at 125 volts. The gels were incubated for 10 minutes in 1X Transfer Buffer (Novex) and blotted to Immobilon-P membranes (Novex) for 1-2 hours at 30 volts. The membranes were incubated in 1X Tris-buffered saline containing Tween 80 (TTBS), supplemented with 5% powdered milk for at least one hour (typically overnight). The blot was rinsed twice with TTBS for 15 minutes, and incubated with primary antibody for 1 hour at a dilution of 1/1000. The blot was rinsed twice with TTBS for 15 minutes before incubating with secondary antibody (alkaline phosphatase conjugated goat anti-rabbit antibody, Promega, Madison, WI) for 1 hour at a dilution of 1/4000. The alkaline phosphatase was visualized by incubating the blot in nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Promega) for 5 to 15 minutes, and the reaction stopped by rinsing extensively in H_2O.

Viral replication was examined by multistep growth analysis on the BHK/UL26 helper line and on BHK cells that did not contain the helper function but were G418-resistant (BHK/C2). Cells (1 x 10^5) were seeded in wells of a 24-well plate and infected with an MOI of 0.1 plaque-forming-units (pfu) per cell. At various times post infection, the infected cells were subjected to three rounds of freeze-

thawing [Tengelsen et al, J. Virol. 67:3470-3480 (1993)] and the lysates were titered on the BHK/UL26 helper line.

To generate cell lines capable of supporting replication recombinant viruses with a deletion and insertion within the UL26 open reading frame, BHK cells were cotransfected with pMON15831a which has the 3.4 kb *Kpn*I fragment of HSV-1 (KOS) 5´ to the SV40 polyadenylation signal (Figure 1) and SV2neo. G418-resistant cells were isolated and shown by Southern blot analysis to contain the HSV-1 *Kpn*I fragment. To determine which cell line would express the UL26 gene products, the cell lines were infected with HSV-2 (MS) to stimulate the UL26 promoter in the cell. HSV-1-specific anti-peptide antisera, generated by inoculating rabbits with the peptide HSVAs-414 conjugated to diphtheria toxin, was used to identify expression of the cellular UL26 gene products (data not shown). This cell line, designated BHK/UL26/8, was used for generation of recombinant viruses. A G418-resistant cell line which was cotransfected with pMON3327 and SV2neo serves as a control and is designated BHK/C2. An additional helper cell line (BHK/UL26 helper) was isolated after the discovery that significant amounts of rescued virus were being generated due to recombination with the *Kpn*I fragment present in BHK/UL26/8. This second line was transfected with plasmid pMON15840 which has the UL26 ORF behind the ICP6 promoter and lacks the large amount of HSV DNA 5´ to the UL26 ORF contained in pMON15831a. Translation from this integrated plasmid began at the methionine at the natural amino acid 10. Candidate cell lines were screened for their ability to support growth of the blue plaque phenotype recombinant virus (see below). A cell line isolated from this latter screening that supports the growth of the UL26 mutant virus was designated the BHK/UL26 helper cell line.

Cell line BHK/UL26/8 was transfected with HSV-1

(strain 17) genomic DNA and plasmid pMON15835 which contains a *Not*I fragment of HSV-1 (strain 17) with a deletion in the protease domain of the UL26 ORF and an insertion of the bacterial β-glucuronidase gene under control of the HSV-1 (strain 17) ICP6 promoter (Figure 1). After cell lysis, the supernatant was serially-diluted on BHK/UL26/8 and blue plaques were identified after 4 to 5 days post infection. The blue plaques were picked and plaque-purified three times. The recombinant virus was designated HSV/UL26/β-gluc. Plaque purification indicated poor segregation between the blue phenotype recombinant virus and a clear plaque phenotype virus which appeared to have a growth advantage, even on the helper cell line.

To determine the genotype and source of the clear plaque virus, DNA amplification was performed on cell-free viral DNA from the mixed culture of blue and clear plaque phenotype viruses. Amplification of a 283 bp fragment indicated the presence of wild-type virus in the stock. The PCR product was digested with *Bsg*I, which cuts the fragment from wild-type (strain 17) DNA, but does not cut the fragment from wild-type (strain KOS) DNA, which is the source of DNA in the helper cell (data not shown). Lack of digestion of the PCR product by *Bsg*I indicated that the wild-type virus was actually a revertant generated by recombination between the blue plaque phenotype virus and the UL26 sequences in the helper cell line. The rescued virus was designated HSV/UL26/res.

In order to generate a more pure stock of HSV/UL26/β-gluc, a new helper cell line (BHK/UL26 helper) was isolated in which the amount of HSV DNA sequence 5′ to the UL26 ORF was eliminated and replaced with the ICP6 promoter region fragment (pMON15840, Figure 1). Propagation of HSV/UL26/β-gluc on this cell line resulted in only the blue plaque phenotype.

Viral DNA from wild-type (strain 17), HSV/UL26/β-gluc

and the rescued virus was digested with *Not*I or *Kpn*I. The digested DNA was analyzed by Southern blot analysis after probing with a restriction fragment containing the full length UL26 open reading frame and 5′ flanking sequences. The results showed the expected pattern of digestion (Figure 2). Wild-type and rescued virus showed the same pattern as expected with both *Not*I (6.3 kb) and *Kpn*I (3.4 kb) digestion (Lanes 1 and 3). Deletion of a small region of the UL26 ORF and insertion of the β-glucuronidase gene resulted in addition of a new *Not*I site (resulting in predicted 4.8 and 4.4 kb fragments) and a new *Kpn*I site (resulting in a 4.0 and 2.1 kb fragments) (Lane 2) in HSV/UL26/β-gluc.

Growth curves were determined for the viruses on the different cell lines. At various times post infection, the cells were harvested and freeze-thawed three times before plating on BHK/UL26 helper cells. The results indicated that HSV/UL26/β-gluc failed to replicate in BHK/C2 cells but grew with wild-type kinetics on the BHK/UL26 helper cell line. The wild-type (strain 17) HSV-1 and the rescued virus replicated to identical titers and at identical rates on both BHK/C2 and the BHK/UL26 helper cell lines (Figure 3).

Since it has been shown by transient transfection experiments in mammalian cells, bacteria and ts1201 that certain mutations in the 5′ region of UL26 fail to cleave the capsid assembly protein [reviewed in Gao et al, J. Virol. 68:3702-3712 (1994)], HSV/UL26/β-gluc was used to infect BHK/C2, BHK and BHK/UL26 helper cells at an MOI of 5. At 18 hours post infection, the cells were lysed in SDS-PAGE sample buffer and proteins separated on a 14% SDS-PAGE gel. After transfer to Immobilon P membranes, the blots were incubated in antisera against the HSV-1 capsid assembly protein. The results are shown in Figure 4. Infection of BHK/C2 cells by HSV/UL26/β-gluc resulted in a

failure to process the capsid assembly protein to a lower molecular weight form. Infection of BHK/helper cells by HSV/UL26/β-gluc showed that the capsid assembly protein was appropriately processed. The rescued recombinant virus (HSV/UL26/res) processed the capsid assembly protein in both cell lines as did wild-type HSV-1 (lanes 2 and 4). The capsid assembly protein was made at normal levels during infection in both helper and non-helper cells but is not cleaved in the non-helper cells. The HSV/UL26/β-gluc recombinant fails to process the capsid assembly protein and has restricted growth.

Female Swiss-Webster mice (12-14 grams, Charles Rivers Laboratories, Wilmington, MA) were inoculated with virus intraperitoneally or subcutaneously with 100 µl volumes. Subcutaneous inoculations were delivered on the dorsal side near the base of the tail after brief CO_2/O_2 treatment of the mice. Virus was resuspended in DMEM containing 5% FBS unless otherwise noted. Food and water were given *ad libitum*. Mice were euthanized if they became moribund due to paralysis.

Mice were inoculated i.p. with 6 x 10^5 pfu (as determined on the helper cell line) of either the wild-type (strain 17) HSV, HSV/UL26/β-gluc, or the rescued virus in a 100 µl volume. As shown in Figure 5, mice infected with wild-type (strain 17) or the rescued virus died by day 7 post infection. All mice infected with HSV/UL26/β-gluc survived. The animals that originally received HSV/UL26/β-gluc were challenged with wild-type HSV-1 (strain 17), i.p., at the same dose given initially. Age- and sex-matched naive mice were also inoculated. One of the HSV/UL26/β-gluc infected mice was found dead about 16 hours post infection with the wild-type virus. Death was probably not related to the virus since it occurred so quickly after infection. The other 9 mice survived the wild-type virus challenge. The naive mice were susceptible

to wild-type virus infection although it took longer for the virus to cause morbidity and mortality (Figure 5).

In a second experiment, mice were inoculated i.p. with ten-fold serial dilutions of HSV/UL26/β-gluc starting at the same inoculum used in the initial experiment. On day 39, the mice were challenged i.p. with 6×10^6 pfu of HSV-1 (strain 17). This dose of wild-type virus was 10-fold higher than that in the initial experiment and resulted in 90% death in the mice that were initially inoculated with DMEM/5% FBS (Table 1, mock-infected set). Again, within 16 hours, 6 mice were found dead. Two of these were in the set that were previously inoculated with 10 pfu of HSV/UL26/β-gluc and 4 were in the set that were previously given 1×10^5 pfu of HSV/UL26/β-gluc. There was a significant difference among the six survival curves ($p<0.02$, log rank test). The data suggests that mice that were inoculated with HSV/UL26/β-gluc survived in a dose-dependent manner (Table 1). The survival curves of the mice receiving the highest dose of HSV/UL26/β-gluc were statistically different from the mock group ($p=0.023$, log rank test).

Table 1.

HSV/UL26/β-gluc	% Survival*
mock	10
6×10^1 pfu	12.5
6×10^2 pfu	30
6×10^3 pfu	60
6×10^4 pfu	50
6×10^5 pfu	83.3

* Survival determined on day 20 after i.p. challenge with 6×10^6 pfu of wild-type HSV-1 (strain 17). N = 10 for all groups except for the 6×10^1 (N = 8) and 6×10^5 (N = 6) due to the early death.

In a third experiment, virus stocks were prepared as previously but were resuspended in DMEM without any FBS. Sets of ten mice were inoculated with DMEM alone or with increasing doses of HSV/UL26/β-gluc by either i.p. or s.q. routes. After one month, all mice were challenged with 10^7 pfu of wild-type virus by i.p. inoculation. Some controls for rapid death included animals that received i.p. media then challenged with i.p. media, HSV/UL26/β-gluc and then media or, HSV/UL26/β-gluc and then challenged with HSV/UL26/β-gluc. None of these animals died during the course of the experiment. None of the experimental animals died within 24 hours of challenge. Of these, 90 animals had received two inoculations of virus and one would expect about 10-12% to have died rapidly. The results with the experimental groups are shown in Figure 6A and 6B. There was a significant difference among the survival curves for both the i.p. ($p<0.01$) and s.q. ($p<0.01$) inoculations (log rank test). Regression analysis shows that there is a dose-dependent effect of HSV/UL26/β-gluc on survival ($p<0.05$, Cochran-Armitage test) for both groups.

It is expected that this virus would have reduced efficiency and reactivate poorly, if at all. The fact that the mutation effects a late gene function suggests that the recombinant virus may be more efficacious in inducing immunity than viruses that have deletions in immediate early or early genes. The assembly-defective HSV/UL26/β-gluc virus is a member of a new class of vaccine candidates with a defect in late gene activity.

It is anticipated that the defect in the essential gene described in an assembly-deficient virus can be incorporated in a virus with other mutations in essential or nonessential genes. Such genes, like ICP47 of HSV-1, may modulate the host's ability to mount an immune reaction to the virus [Hill et al, Nature 375:411-415 (1995); Früh

et al, Nature 375:415-417 (1995)].

The vaccines of the present invention can be of a lyophilized form or suspended in a pharmaceutically-acceptable carrier. Suitable suspensions can include phosphate buffer, saline, glucose, inactivated serum, excipients, and adjuvants. The vaccine can be prepared and used according to standard techniques well known in the art [reviewed in R. L. Burke, Seminars in Virology, 4:187-197, (1993)]. The effective dose may also be determined by standard techniques well known in the art. Generally, vaccines are formulated in a suitable sterilized buffer and administered by intradermal, intramuscular, or subcutaneous injection at a dosage of between 10^3 and 10^9 pfu/kg. The vaccine can also be formulated for oral or ocular administration in vehicles known in the art.

The foregoing detailed description is given to facilitate clearness of understanding only, and no unnecessary limitations are to be understood therefrom, as modifications within the scope of the invention will be obvious to those skilled in the art.

What is claimed:

1. A vaccine comprising an assembly-deficient herpesvirus.

2. The vaccine of Claim 1 wherein said herpesvirus contains an inactivated form of an essential protease gene.

3. The vaccine of Claim 2 wherein said essential protease gene is required for the processing and assembly of immature, noninfectious capsid particles into mature, infectious capsid particles.

4. The vaccine of Claim 1 wherein said herpesvirus is selected from HSV-1, HSV-2, HCMV, SCMV, VZV, EBV, HHV-6, HHV-7, HHV-8, PRV, BHV and EHV.

5. The vaccine of Claim 4 wherein said herpesvirus is HSV-1 or HSV-2.

6. The vaccine of Claim 4 wherein said herpesvirus is HSV-1.

7. The vaccine of Claim 3 wherein said essential protease gene is selected from HSV-1 UL26, HSV-2 UL26, and HCMV UL80.

8. The vaccine of Claim 7 wherein said essential protease gene is HSV-1 UL26.

9. The vaccine of Claim 2 wherein said essential protease gene is inactivated by a method selected from deletion, insertion, substitution of DNA, and any combination of deletion, insertion, or substitution of DNA.

10. The vaccine of Claim 9 wherein said essential protease gene is inactivated by deletion of viral DNA and insertion of nonviral (heterologous) DNA.

11. The vaccine of Claim 1 comprising between about 10 and about 10^6 plaque-forming units of said herpesvirus.

12. The vaccine of Claim 1 wherein said assembly-deficient herpesvirus comprises the strain designated HSV/UL26/β-gluc.

13. A method of manufacturing a vaccine of Claim 1 comprising an assembly-deficient herpesvirus, by preparing stocks of said herpesvirus in a recombinant cell line capable of generating properly-assembled virus, and suspending said virus in a pharmaceutically-acceptable carrier.

14. The method of manufacturing a vaccine of Claim 13 wherein said essential protease gene is an HSV-1 UL26 gene.

15. The method of Claim 13 wherein said vaccine comprises the strain HSV/UL26/β-gluc.

16. The method of Claim 13 wherein said cell line is mammalian.

17. The method of Claim 16 wherein said cell line supports replication of said herpesvirus.

18. The method of Claim 17 wherein said cell line is the cell line designated BHK/UL26/8.

19. The method of Claim 17 wherein said cell line comprises the cell line designated BHK/UL26 helper.

20. A use of an assembly-deficient herpesvirus in a preparation of a vaccine.

21. A method of immunizing a mammal against a herpesvirus by administering a vaccine of Claim 1 in a pharmaceutically-acceptable carrier.

22. The method of Claim 21 where the mammal is selected from human, monkey, cow, horse, sheep and pig.

23. The method of Claim 22 where the mammal is human.

24. A mutant herpesvirus containing an inactivated form of an essential protease gene required for the processing and assembly of immature, noninfectious capsid particles into mature, infectious capsid particles, with said essential protease gene inactivated by deletion of viral DNA and insertion of nonviral (heterologous) DNA.

25. A mutant virus according to Claim 24 wherein said virus is selected from HSV-1, HSV-2, HCMV, SCMV, VZV, EBV, HHV-6, HHV-7, HHV-8, PRV, BHV and EHV.

26. A mutant virus of Claim 25 wherein said essential protease gene is HSV-1 UL26.

27. A mutant virus of Claim 24 wherein a portion of said essential protease gene is deleted and replaced by a nonviral (heterologous) DNA segment comprising a reporter gene under the control of an inducible herpesvirus HSV-1 promoter.

28. A mutant virus of Claim 27 wherein said reporter gene is selected from gusA encoding beta-glucuronidase, lacZ encoding beta-galactosidase, phoA encoding alkaline phosphatase, gfp encoding green fluorescent protein, and aeq encoding aequorin.

29. A mutant virus of Claim 28 wherein said reporter gene is the gusA gene encoding *E. coli* beta-glucuronidase.

30. A mutant virus of Claim 27 wherein said inducible herpesvirus promoter is the HSV-1 ICP6 (UL39) promoter.

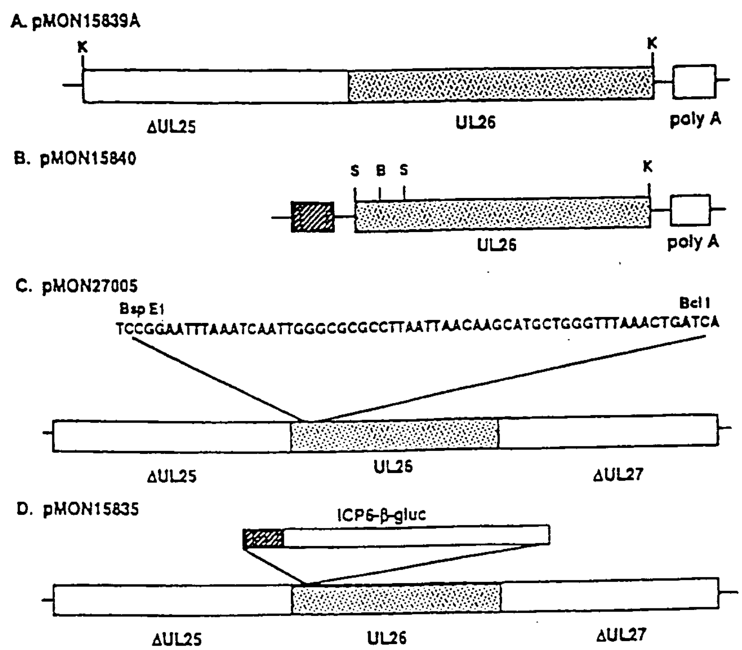
31. A recombinant host cell line expressing an essential herpesvirus protease gene under the control of an inducible non-protease promoter.

32. A recombinant host cell line of Claim 31, wherein said host cell line is from a rodent source.

33. A recombinant host cell line of Claim 32, wherein said host cell line is BHK-21.

34. A recombinant host cell line of Claim 31 wherein said inducible non-protease promoter is the HSV-1 ICP6 (UL39) promoter.

35. A method of making mutant herpesviruses of Claim 24 by introducing said virus into a recombinant host cell line and recovering mature viral particles harboring the mutant viral genome.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K
K
ΔUL25
UL26
poly A
B. pMON15840
S B S
K
UL26
poly A
C. pMON27005
Bsp E1
Bcl I
TCCGGAATTTAAATCAATTGGGCGCGCCTTAATTAACAAGCATGCTGGGTTTAAACTGATCA
ΔUL25
UL26
ΔUL27
D. pMON15835
ICP6-β-gluc
ΔUL25
UL26
ΔUL27

Fig. 1

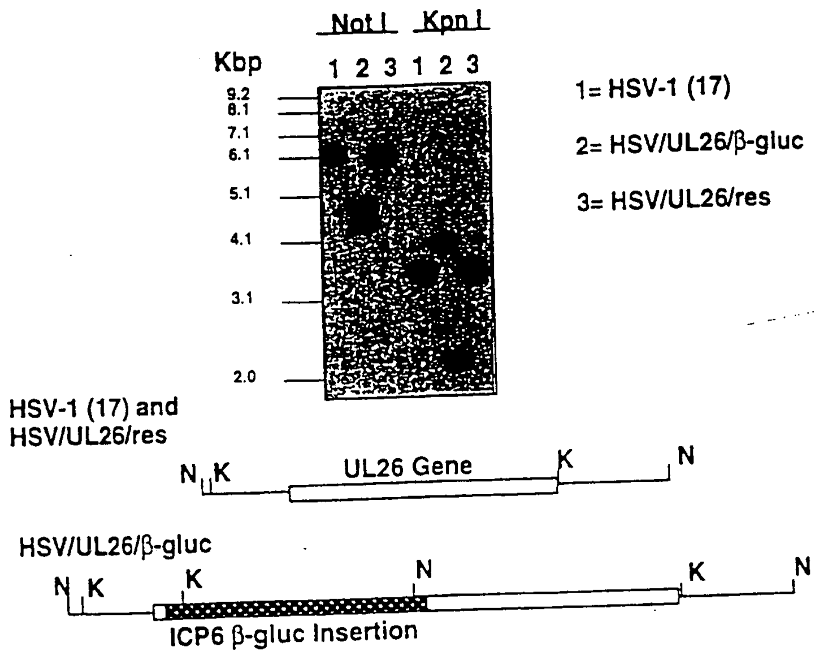

Not I
Kpn I
Kbp
1 2 3 1 2 3
9.2
8.1
7.1
6.1
5.1
4.1
3.1
2.0
1= HSV-1 (17)
2= HSV/UL26/β-gluc
3= HSV/UL26/res
HSV-1 (17) and
HSV/UL26/res
N
K
UL26 Gene
K
N
HSV/UL26/β-gluc
N
K
K
N
K
N
ICP6 β-gluc Insertion

Fig. 2

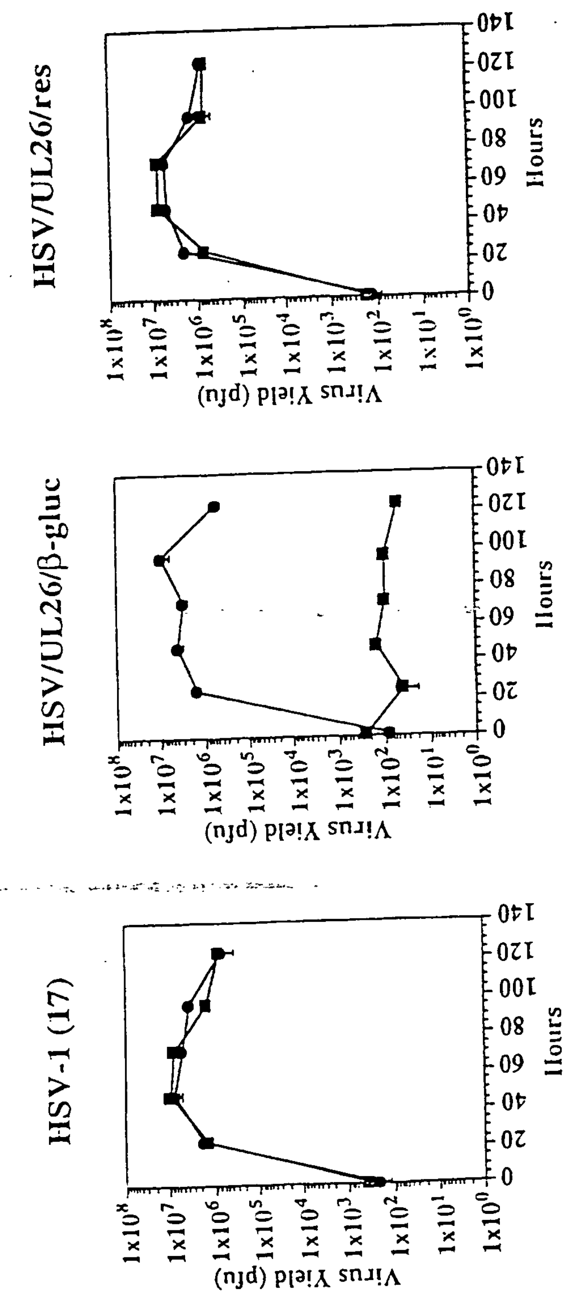

HSV-1 (17)
HSV/UL26/β-gluc
HSV/UL26/res
Virus Yield (pfu)
Hours
1x10^8
1x10^7
1x10^6
1x10^5
1x10^4
1x10^3
1x10^2
1x10^1
1x10^0
0
20
40
60
80
100
120
140

Fig. 3

1= Mock

2= HSV-1 (17)

3= HSV/UL26/β-gluc

4= HSV/UL26/res

M= MW$_r$ Markers

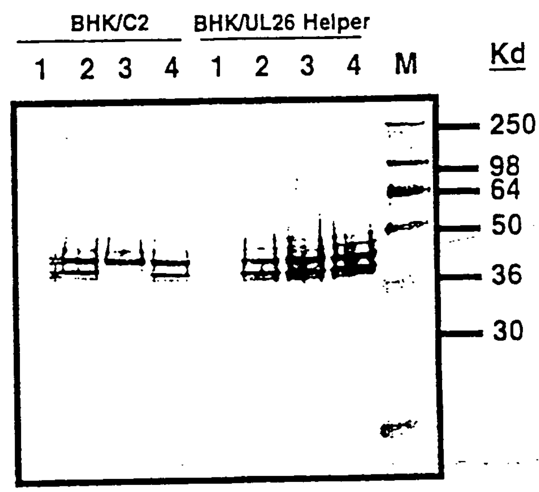
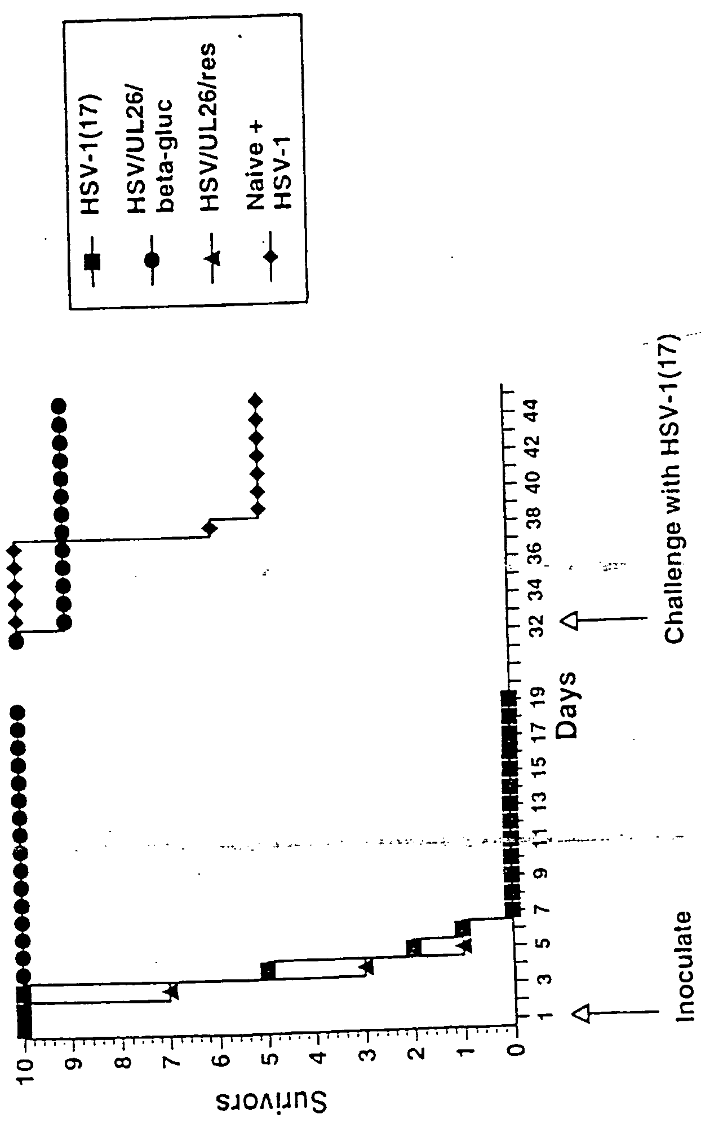


Fig. 4

Fig. 5


HSV-1(17)
HSV/UL26/ beta-gluc
HSV/UL26/res
Naive + HSV-1
Survivors
0 1 2 3 4 5 6 7 8 9 10
1 3 5 7 9 11 13 15 17 19 32 34 36 38 40 42 44
Days
Inoculate
Challenge with HSV-1(17)

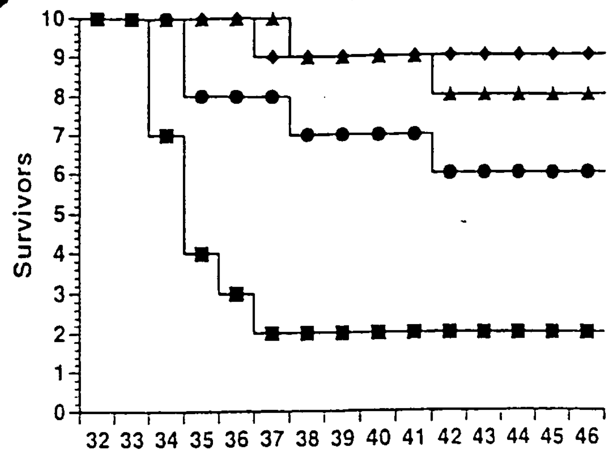

Survivors
10
9
8
7
6
5
4
3
2
1
0
32 33 34 35 36 37 38 39 40 41 42 43 44 45 46

Fig. 6A

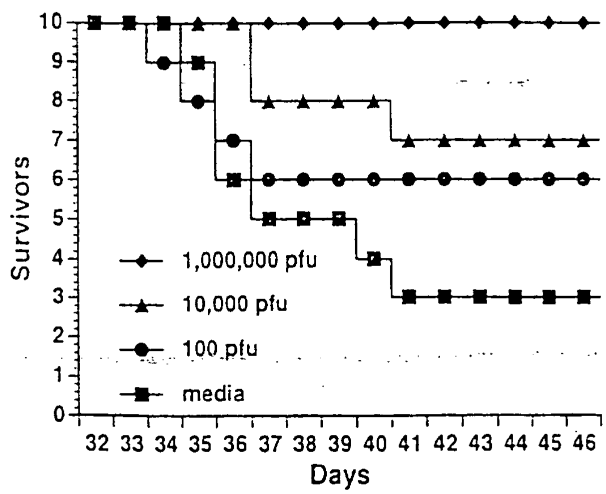

Survivors
10
9
8
7
6
5
4
3
2
1
0
1,000,000 pfu
10,000 pfu
100 pfu
media
32 33 34 35 36 37 38 39 40 41 42 43 44 45 46
Days

Fig. 6B

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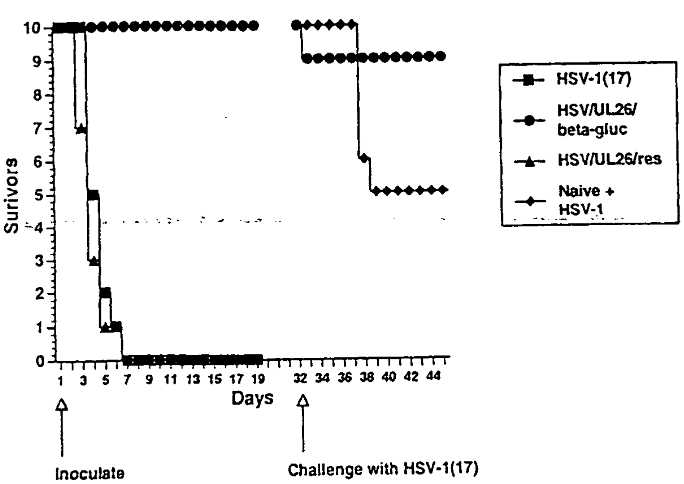

(57) Abstract

A vaccine is described which comprises an assembly-deficient herpesvirus. The mutant herpesvirus is capable of infecting and undergoing DNA replication in the cells of a susceptible mammal, but is defective in capsid assembly and formation of mature virion particles. The assembly-deficient herpesvirus is avirulent and capable of generating a protective immune response in a vaccinated mammal.

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International Application No
PCT/US 97/14192

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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 96 38551 A (MERCK & CO. INC.) 5 December 1996 see page 1, line 18 - page 2, line 2 see page 6, line 1 - line 6 see page 8, line 18 - line 22 see page 8, line 29 - page 9, line 10 see page 23, line 18 - line 23 ---	24-26, 31,35
X Y	WO 92 13943 A (SMITHKLINE BEECHAM BIOLOGICALS S.A.) 20 August 1992 see page 5, line 34 - page 6, line 7; claim 11; example 6 ---	1-14,16, 17,20-23 15,18, 19,24-35

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[X] Further documents are listed in the continuation of box C.

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1

Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA	Authorized officer
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Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	M.F. AL-KOBAISI ET AL.: "The Herpes simplex virus UL33 gene product is required for the assembly of full capsids" VIROLOGY, vol. 180, 1991, pages 380-388, XP002057787 see the whole document	1-35
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